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Protein-Protein Interactions

Pathophysiological and Therapeutic Aspects: Volume II

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Pathophysiological and Therapeutic Aspects: Volume II

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Dedicated to all our teachers, mentors, and parents for their invaluable guidance and unconditional support throughout our research careers.

Preface

Inside the cell, a complex network of dynamic molecular interaction takes place without interruption. Such molecular interactions are like the strings pulled by the puppeteer in order to perform the act of life in a precise and accurate manner. In this act of sustenance, proteins are the major puppeteers that maintain and balance the biological equilibrium with the help of intricate protein–protein interactions (PPIs), as they are involved in diversified activities including genetic transfer, cell proliferation, and apoptosis of a cell. Owing to this fact, over the last two decades, the quantification/prediction of individual PPIs and protein interaction networks has been of prime focus for the researchers across the globe.

The identification of PPIs has led to promising opportunities in expanding the knowledge of human physiology, as well as in the development of therapeutic strategies in cases of life-threatening diseases. It is important to evaluate such interactions as they predetermine the phenotype, behavior, and stimuli response of biological systems under both physiological and pathophysiological conditions. PPIs are directly involved in biological mechanisms; hence, utilizing PPIs as targets for drug development has been an efficient process. However, with increasing cases of resistance against broad-spectrum drugs, and ever-evolving conditions of pathogenic microorganisms has created a bottleneck in the field of drug development. Although over 645,000 disease-associated PPIs are known in the human interactome, only 2% of these leads have been explored as therapeutic targets. Therefore, deep understanding of novel PPIs and their mechanism of interaction is quintessential. Advent of high throughput experimental and computational techniques that are able to decode the criteria followed by the proteins during the course of their interactions with other protein partners has aided immensely in elucidating the protein interaction networks and their drug targets. The previous volume in the series focused on understanding the basis of protein–protein interaction and providing a comprehensive knowledge of complex experimental and computational tools used to elucidate and predict missing connections in PPI network. While this book provides a comprehensive account of protein–protein interaction that takes place in conditions such as genetic disorders, cancer, immune disorders, pathogenic infections, and some novel accounts of how PPI inhibitors against such interactions have been developed over the years.

This book comprises of seven chapters of which the first five chapters discuss the plethora of research done in the field of PPI identification in cases of various diseases while the last two chapters account for different ways of designing, types, and examples of PPI inhibitors against some popularly known protein–protein interactions involved in the pathophysiology of a human body in diseased state. Chapter 1 presents an overview of various protein-related disorders, where mutations play a vital role in the formation of diseases such as cystic fibrosis, diabetes, GPCR-related disorders due to aberrant protein–protein interactions. Chapter 2 elucidates the comprehensive knowledge on protein–protein interactions involved at different stages and types of cancer malignancies and discusses the potential of single cell profiling and protein interaction networks for the development of novel therapeutic candidates. Chapter 3 provides an outline about the basic principles of neurodegeneration, its symptoms and pathophysiology with the major focus on protein interaction underlying the neurodegenerative disorders. Moving further, Chap. 4 outlines the pathophysiology and underlying protein interactions involved during immune system and inflammatory disorders such as inflammatory bowel syndrome, multiple sclerosis, and diabetes. In addition to this, it gives a brief account of the fundamentals of inflammation and immune disorders. Chapter 5 dissects the molecular interaction between pathogen and host proteins that are essential for the pathophysiology of a pathogenic infections and furnishes the details of various host–pathogen interaction involved at each step of infection. Chapter 6 addresses the basis of drug design and various experimental and computational methodologies utilized till date for identifying drug targets that are efficient and cost-effective. Chapter 7 is devoted to small molecule inhibitors of PPIs and renders a detailed account of small molecules that are already being used/proposed as potential PPI inhibitors.

This book provides a detailed information of protein–protein interactions responsible for digression of diseases in humans and correlates it with the fundamental concepts of PPI interaction discussed in the previous volume. Further comprehensive information of various approaches taken in the field of drug designing against such PPIs together with their examples and current status has been presented in this book. This book aims to deliver a detailed and meticulous understanding of disease-related PPIs and their inhibitors known till date to students and researchers working in the fields of protein chemistry and biology.

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Protein Networks in Human Disease

1

1.1 Introduction to Genetic Basis of Disease

The genetic code provides a blueprint for the development and survival of an organism. The human genome is roughly made up of three billion base pairs of DNA. The genome encodes for approximately 22,000 gene coding RNAs and proteins needed for the development and proper functioning of the human body. In addition, over 14,000 noncoding genes or "pseudogenes" have been identified in the human genome. These genes are usually nonfunctional copies of functional genes that have lost their protein-coding ability over the course of evolution. The noncoding regions of the genome participate in regulating the protein-coding regions for maintaining a healthy state (Cheetham et al. 2020). Moreover, pseudogenes promote recombination events and are native sites for mutation, which lead to diseases like Gaucher disease, Down syndrome, and cancer. Mutations and variations are the key factors that distinguish individuals and populations; and are also responsible for autoimmune and pathogenic disorders. Mutations can be heritable change in the DNA sequence where the change could refer to somatic cell division or germline inheritance (Shendure and Akey 2015). In contrast, variants refer to differences in the gene/genome from the reference gene. Variations are common mutations (disease-associated) that are generally defined at the population level. At the population level, the variant version of the gene sequence is referred to as "alleles." Allelic differences are the potent cause of polymorphism. The variations can either be benign (nonfunctional) or disease-causing. Human diseases include a wide variety of genetic changes. These include chromosomal imbalances, single-gene disorders, point mutations and indels, complex disorders, and epigenetics.

Chromosomal imbalances or aberrations can occur due to numerical or structural abnormalities. An average human somatic cell consists of DNA compactly arranged into 23 pairs of chromosomes (22 + XX/XY), maintaining the diploid state of the cell. However, in some cases, the number of chromosomes are not an exact multiple of the haploid set, and such condition/state is known as aneuploidy. Aneuploidy

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generally occurs when a gamete contains fewer or a greater number of chromosomes than usual due to nondisjunction in cell division. Aneuploidies are lethal and lead to severe developmental abnormalities (Orr et al. 2015). Based upon the total number of chromosomes, two most common conditions of aneuploidy known are monosomy (2n − 1) and trisomy (2n + 1). Aneuploidy can either be autosomal or abnormal number of sex chromosomes (Table 1.1). At the same time, structural abnormalities result from DNA damage, which leads to chromosomal breaks. The error might occur at multiple levels of DNA-related mechanisms such as replication, DNA repair, DNA packaging, and recombination. The cytogenetic study has revealed that the underlying causes of structural abnormalities in many cases are microdeletions, microduplications, and copy number variants (CNVs) (Chunduri and Storchová 2019). Some of the associated syndromes due to genetic anomaly have been listed in Table 1.1.

Single-gene disorders are based on the genotype at a single locus where heredity is in accordance with the Mendelian laws of segregation, independent assortment, and dominance. These disorder genotypes are classified as dominant and recessive and are either autosomal or sex chromosome-related (Chial 2008). Genotype modifications can occur due to point mutations or indels leading to severe functional abnormalities. Some pathogenic single-gene disorders are hemophilia, cystic fibrosis, phenylketonuria, etc., (Table 1.1). In addition to modifications in the nuclear genome, aberrations in the mitochondrial genome also led to severe human abnormalities. The mitochondrial genome is responsible for expressing critical electron transport chain enzymes and other biological processes that occur inside the mitochondria. It is always inherited via cytoplasmic inheritance, hence inherited only from the mother. Therefore, if the mother is affected, the whole pedigree is also affected. Several conditions, such as Leber's hereditary optic neuropathy (LHON) and Leigh syndrome are considered as mitochondrial genome-associated diseases. Epigenetics is also a central genetic feature responsible for diseases and phenotypic changes seen in humans and other organisms. DNA undergoes chemical modifications in a normal cell, and the study correlating such chemical changes is known as epigenetics. The chemical changes include methylation, demethylation, histone modifications, chromosome imprinting, etc. The DNA contains specific sites for these processes to occur, for example, DNA methylation occurs at the CpG islands with the help of methylase enzymes. Moreover, the chemical modifications affect/regulate the DNA replication and packaging processes. A recent report by Jackson et al. had comprehensively reviewed the relation between epigenetics and genetic disorders (Jackson et al. 2018). Understanding the genetic basis of diseases provides an insight into the intricate conversations at the genome level and how they affect the phenotypic level. It has led to a better diagnosis, prognosis, and treatment of genetic disorders in several cases. Apart from the genetic basis, it is quintessential to understand and analyze the protein expression, structure-function relationships, and their binding interactions as they are working horses of the living system, and phenomenally contributes to the pathophysiological conditions of the living organism (Poluri et al. 2021d).

Table 1.1 Examples of diseases due to various genetic anomaly (Jackson et al. 2018; Orr et al. 2015)

Common name	Genetic anomaly	Symptoms
Aneuploidy		
Patau syndrome	Trisomy 13	Intellectual disability; motor disorder; microphthalmia; meningomyelocele; polydactyly; cleft palate; kidney and heart defects
Edwards syndrome	Trisomy 18	Kidney malformations; structural heart defects at birth; omphalocele; esophageal atresia; intellectual disability; arthrogryposis; microcephaly; micrognathia; cardiac and CNS abnormalities
Down syndrome	Trisomy 21	Moderate intellectual disability; characteristic facial appearance; delayed physical growth; short stature; hearing and vision disorder; leukemia
Triple X syndrome	Trisomy X	Tall stature; seizures; delayed speech development, language, and motor skills; skeletal anomalies; behavioral and emotional difficulties
Jacob's syndrome	47, XYY	Autism; emotional and behavioral issues; delayed speech; motor skills; hypotonia; and involuntary muscle movement
Klinefelter syndrome	47, XXY	Smaller male genital organs; taller stature; low testosterone levels; absent or delayed puberty; gynecomastia; reduced facial and body hair; infertility; learning disabilities; speech and language delay
Microdeletion/microduplication-related disorders		
Di George syndrome	22q11.2 deletion	Susceptible to infections and autoimmune disorders; heart defect; cyanosis; characteristic facial features; learning difficulties; hypocalcemia, renal and skeletal abnormalities
Williams syndrome	Chromosome 7 partial deletion	Mild intellectual disability; problems with coordination; delayed development; hypercalcemia; supravalvular aortic stenosis; distinctive "elfin" facial appearance
Smith–Magenis syndrome	Short p-arm of chromosome 17	Moderate intellectual disability; disrupted sleep patterns; behavior issues; short flat head; broad nasal bridge
Cri-du-chat syndrome	5p15.2 CTNND2	Cat-like cry; microcephaly; palate abnormalities; severe psychomotor skills; severe intellectual disability
Single-gene disorder due to point mutations		
Autosomal dominant		
Glut1 deficiency	SLC2A1	Mental confusion; drowsiness; rapid head and eye movement; hemiparesis; microcephaly
Osteogenesis imperfecta	COL1A1 or COL1A2	Bone deformities; barrel-shaped chest; discoloration in the eye; weak muscles; loose joints
Autosomal recessive		
Phenylketonuria	PAH	Distinct odor; neurological problems; eczema; fair skin and blue eyes; no or low melanin production
Sickle-cell anemia	HBB	Swelling in hand and feet; delayed growth; vision problems; anemia; paleness; breathlessness

(continued)

Table 1.1 (continued)

Common name	Genetic anomaly	Symptoms
X-linked dominant		
Hemophilia A	F8	Arthritis; frequent nosebleeds; blood in urine; excessive bleeding during injury; bruises
Duchenne muscular dystrophy	DMD	Scoliosis; breathlessness; problems with memory and learning; contractures
X-linked recessive		
Fragile X syndrome	FMR1	Trembling hands; balance problems; numbness in hands and feet; cognitive issues; memory loss; reduced fertility
Rett syndrome	MECP2	Hypotonia; mobility problems; delay of speech; loss of memory; difficulty in eating; seizures; irregular breathing pattern
Single-gene disorder due to nucleotide repeat expansion		
Huntington disease	HTT	Memory lapses; depression; clumsiness; mood swings; difficulty speaking; fidgety movements
Myotonic dystrophy type 1	DMPK	Myotonia; weak muscles; cataracts; cardiac conduction defects; fatigue; difficulty in swallowing

1.2 Classical Protein Complexes in the Cell

Proteins are molecular machineries that perform all the biological processes and balance the homeostasis in human body. Proteins work in association with each other and a variety of biological molecules. They act as a relay between cells and organelles and transmit biological signals. One of the classic examples of protein complexes involved in biological processes is RNA polymerase and ribosome-mediated transcription and translation. The dynamic and ordered protein complexes are the molecular essence of cellular machinery (Meng et al. 2021). Coordinated gene expression, translation, transport, and localization of proteins mediate the complex/supermolecule formation. Moreover, the protein-protein complex formation is orchestrated by protein assembly code and protein-folding dynamics. The three-dimensional structure and presence of specific amino acids and/or motifs govern the sensitivity and specificity of interactions and modulate the cellular response of the PPI interactome (Gavin and Superti-Furga 2003). Protein–Protein Interactions (PPIs) are defined by eight characteristics: (1) dynamic behavior, (2) multiple interacting partners, (3) cooperativity, (4) protein-folding states, (5) presence of multiple interfaces, (6) reversible nature of the interaction, (7) hotspot regions (binding residues/scaffolds/motifs), and (8) modularity (Keskin et al. 2008). To date, the most understanding of PPIs comes by factoring in proteins as rigid molecules.

In general, this is not the case, as proteins are inherently dynamic, and the dynamicity offers an insurmountable range of interaction possibilities with their

respective partners. Nuclear magnetic resonance spectroscopy (NMR) and cryo-electron microscopy (Cryo-EM) have proven to be valuable techniques in understanding the dynamic behavior of protein–protein interactions over the recent years (Poluri et al. 2021a). Through the years of development in proteomics, scientists have identified numerous modular-binding domains, scaffolds, and motifs for mapping protein–protein interactions. High-throughput techniques such as yeast two-hybrid (Y2H), microarray, mass spectrometry (Poluri et al. 2021a), and NMR spectroscopy have produced a vast amount of data and literature on PPIs involved in molecular processes (Barabasi and Oltvai 2004). However, the protein molecular interactions in a cellular context are far more complex than elucidated via biophysical and proteomic techniques (Gavin and Superti-Furga 2003). In a cell, the association of one protein does not always happen in binary interactions, i.e., a protein can have multiple binding partners. The mutagenicity introduces several levels of complexity and regulative measures on the activity of the protein and its interacting partners. Some classic examples of such regulation are the allosteric regulation of protein kinases during GPCR signaling and feedback loop. Binding cooperativity plays a significant role in the modulation of PPIs. Cooperativity is highly affected by conformational changes and mutations. Loss or gain of function mutants is an important deciding factor of PPIs' biological activity.

Apart from their dynamic characteristics, numerous other factors influence their interactions. Factors such as post-translational modifications, presence of ATP/chaperone molecules, protein folding, pathological states, localization, and their binding partners are some of those that affect protein dynamicity and their interactions in a grave manner (Keskin et al. 2008). Moreover, as protein complexes often interact with one another, these factors are difficult to assess via classical techniques that focus on deciphering binary interactions. Understanding these complex interactions is an integral part of mapping of the signaling pathways and the protein interaction networks. For example, initiation of transcription involves binding RNA polymerase II (RNA pol II) with the promoter region present upstream of the gene sequence. However, this process is not a one-step process as the whole RNA pol II-promoter binding is orchestrated and monitored by a group of upstream proteins such as transcription factors, repressor-binding factors, and enzymes, which bind to RNA pol II thus aiding in the mRNA formation (Hager et al. 2009; Yamamoto et al. 2004). Similarly, DNA duplication during the S phase of the cell cycle is mediated under the tight control of molecular checkpoints patrolled by cyclin-CDK protein complexes in association with their upstream and downstream mediators (Longhese et al. 2003). Modularity is also an essential attribute of protein complexes as it defines the PPI complexity and the differential function. A module is defined as a complex of two protein molecules (Poluri et al. 2021e). For example, Youtiao, a scaffolding protein, binds with NMDA receptor and potassium ion channels and facilitates their binding to protein kinase A and protein phosphatase 1, respectively (Marx et al. 2002; Park et al. 2001). Until now, a wide array of techniques have been employed to understand PPIs. This rapid collection of PPIs led to the development of libraries and the construction of protein interaction networks

(PINs) which provided a better perspective for evaluating cellular organization (Meng et al. 2021; Sarkar and Saha 2019).

1.3 Characterization of Disease-Related Mutations at Protein Interaction Interfaces

Genetic alterations detected in the human population result from single-nucleotide variants (SNVs). Around 58% of SNVs are responsible for single amino acid variants leading to altered protein structure and function. Analyzing the effect of these alterations on protein–protein interactions is crucial. Both experimental and computational methods are utilized to understand the molecular functions of the protein variants. Experimental methods such as X-ray crystallography, NMR, Y2H, and other proteomic analysis have been described elsewhere (Poluri et al. 2021a). Apart from experimental techniques, computational methods that are used to estimate the phenotypic effect of the variants include: SIFT (Sim et al. 2012; Ng and Henikoff 2003), CADD (Yu and MacKerell 2017; Zhao et al. 2020), PMut (López-Ferrando et al. 2017), etc. Computational methods provide cost-effective and less time-consuming alternatives to experimental analysis to characterize disease-related mutations at protein surfaces (Poluri et al. 2021b). Several studies have revealed the effect of mutations on protein–protein interactions. As explained in the above section, the disease-causing mutation at the PPI interface can induce both physiological and geometrical changes. In the following sections, how genetic mutations lead to severely diseased states concerning some necessary mutation-related diseases; and various diagnosis and treatment processes identified till now by utilizing the information from the above-explained alterations in the genome and proteome of the human cell will be presented in detail.

1.4 Role of Protein Interactions in Disease

Understanding the eminence of protein interactions is essential for investigation of molecular mechanisms both in physiological and pathological states. The progression and clinical symptoms of all pathological states are dependent on PPIs and protein interactome. These interactions can either result from genetic anomalies (in cancer, neurodegenerative diseases) or competitive/noncompetitive inhibition of cellular protein complexes by foreign protein agents (Host–pathogen interactions). As proteins are the primal agents of biological function, the systematic study of interactions is important for improvement of biomedical applications. Protein interaction networks play an important role in deciphering the relationship between the structure and function of PPIs (Yeger-Lotem and Sharan 2015). As mentioned earlier, diseased state is often led by mutations affecting the PPI interfaces and functionality of the protein. The protein interaction network together with the gene-level studies can be helpful in deciphering the molecular basis of diseased state and further development of precise treatment and prevention

protocols. The first systematic probing of mutation led effects on PPIs in a diseased state was reported by Zhong et al. (2009). Later in 2012, Wang et al. evaluated the effect of disease-causing mutations using a fully resolved PPI network and found that interaction interfaces are the key hotspot for in-frame mutations leading to the pathological state (Wang et al. 2012). Over the years, numerous databases and bioinformatic tools have been developed for storage and development of novel protein interaction networks with respect to physiological and diseased states (Poluri et al. 2021b). Databases such as TCGA (The Cancer Genome Atlas) (Tomczak et al. 2015), cBioPortal (Gao et al. 2016a), HPIDB (Host–pathogen interaction database) (Ammari et al. 2016) are key libraries of gene expression data, and protein interaction networks isolated from the clinical samples. These databases are also important in understanding novel protein–protein interactions and the complex nature of the human body in a diseased state. The application of PPI networks focuses on four areas: (1) identification of disease-associated gene/protein, (2) correlation of network properties and diseased state, (3) disease-associated subnetworks, and (4) network-based classification of diseases (Sevimoglu and Arga 2014; Poluri et al. 2021c).

Studying PPIs and associated pathways have revealed key findings with respect to diseased states. Firstly, disease-related genes encode highly connected proteins and secondly, they tend to cluster together (Safari-Alighiarloo et al. 2014). The identification of disease-associated PPIs provides us the ability to recognize potential therapeutic targets and plays a major role in prediction of genotype-phenotype associations. These associations are useful in gaining knowledge about novel diseases, and also fast track the identification of therapeutic agents. A classic example of how the PPIs have helped in development of diagnostic, therapeutic and preventive measures against a disease can be observed in the case of the recent coronavirus outbreak. The prerequisite literature on viral genome and genes associated with its clinical symptoms gave an edge in the assessment of the outbreak and led to a fast-track development of vaccines, therapeutic protocols, and testing kits against COVID-19. PPIs and protein interaction networks have unraveled the molecular basis of complex diseases as well. Several complex diseases are a result of complicated protein–protein interactions. Complete understanding of complex diseases such as cancer and autoimmune diseases involving multiple factors and association of genotype to phenotype implications is a difficult task. However, thorough investigation of genes involved in such complex diseases has given some information on the molecular mechanisms involved in their progression (Safari-Alighiarloo et al. 2014). Traditional approaches have not been quite promising in uncovering the molecular mechanisms involved in cancer, as they focus on a single gene or protein. However, the prediction of cancer-specific mechanisms by protein interaction networks provides a much better idea of the disease. Sun and Zhao, utilized the information on gene expression and protein interaction networks involved in prostate cancer that led to development of markers for detection of aggressive and nonaggressive states of prostate cancer (Sun and Zhao 2010). Similarly, a study in 2009, combined the breast cancer gene expression data together with human PINs to unravel the biomarkers of breast cancer prognosis (Taylor et al. 2009).

1.5 Impact of Protein Interaction Networks on Analysis of Disease Genes: A Case Study

COVID-19 has been a devastating health crisis of the twenty-first century. It is caused by a novel coronavirus called SARS-CoV-2. For the past 3 years, COVID-19 has caused insurmountable social and economic disruption globally. The associated mortality and morbidity have been one of the major concerns for the scientific community since 2019. The rate of transmission and a variety of transmissible factors caused the disease to take the shape of a pandemic. The crisis led to the setting up of several initiatives for better understanding of the virus and disease, and for formulation of therapeutics and preventive drugs. High-throughput genome sequencing and PPI networks played an essential role in impactful targeting of the disease. SARS-Cov-2 is a member of β-coronavirus family and is related to SARS-Cov and MERS-Cov at the structural level (Wu et al. 2020a). The SARS-Cov-2 capsid is made up of structural proteins/NSPs (nucleoprotein, membrane, spike, and envelope protein), nonstructural proteins (RNA-dependent RNA polymerase) and a phospholipid envelope (Khedkar and Patzak 2020). The similarity with other coronaviruses provided an upper hand in speeding up the development of therapeutics. For example, spike protein which is also expressed by SARS-Cov and MERS-Cov was already known to interact with human angiotensin-converting enzyme (ACE2) receptors (Li et al. 2006). This served as a premiere target for treatment of COVID-19 in the initial phase of the pandemic. Similarly, identification of NSPs such as RNA-dependent RNA polymerase (RdRp) and 3C-like protease (3CLpro) and their known functions helped in drug development for inhibition of viral replication. Remdesivir which is an adenosine analog is one of the prime examples of this approach (Choy et al. 2020). Moreover, a clear understanding of pathophysiology of SARS-CoV infections made drug discovery and vaccine development an easier process (Fig. 1.1).

1.5.1 Potential Therapeutic Drug Targets in COVID-19

Clinical identification of symptoms and pathological causes of a disease are utilized to establish diagnostic and therapeutic methods. Increasing complexity of diseases and drug pathways has called for change in traditional approaches to drug interventions and strategies. Rather than following the "one drug one target" approach, researchers nowadays are more focused on investigating the impact of drug-induced effects of the molecular networks that also accounts for the synergistic effect of multiple drug treatments (Mestres et al. 2009; Janga and Tzakos 2009). Network-based methods have proven to be highly effective in such cases (Peters 2013). SARS-Cov-2 contains a variety of protein interactions that can be targeted for development of drugs. A concise information of therapeutics developed till date against SARS-Cov-2 and human protein interactions are enlisted in Table 1.2, which either target the viral entry or the viral replication process.

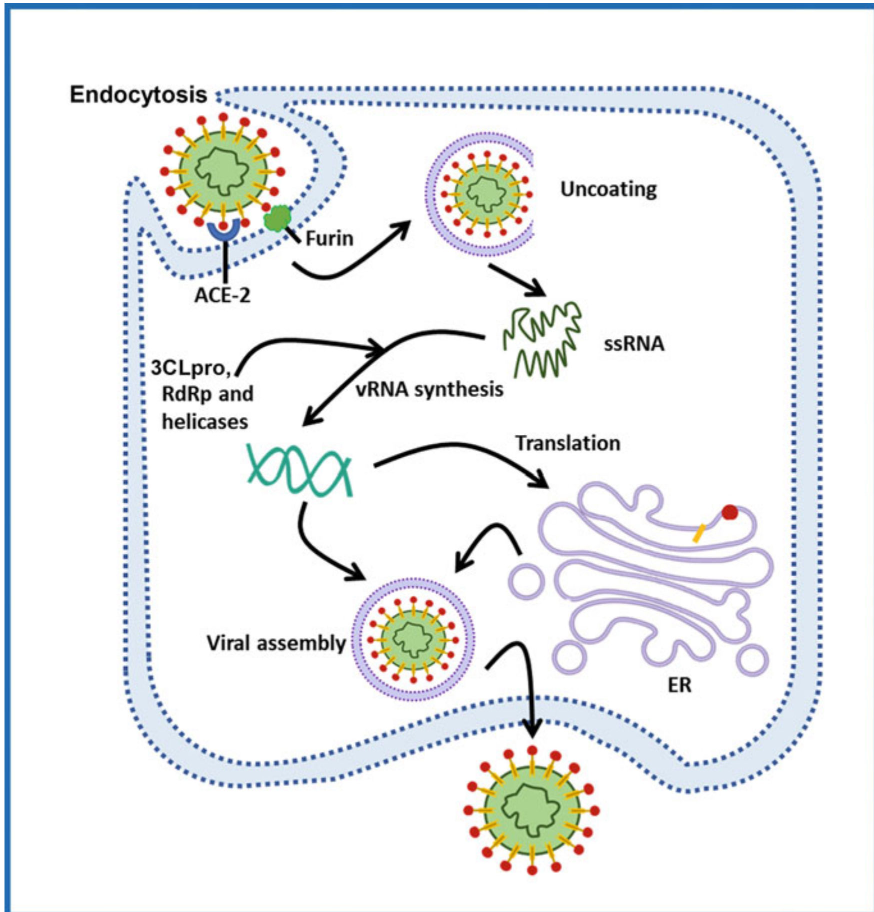

Fig. 1.1 Pathophysiology of SARS-CoV-2 infection. SARS-CoV-2 viral envelope consists of several structural proteins such as spike (S), envelope (E), and nucleoprotein (N). The initiation of SARV-CoV-2 infection is mediated by interaction of S protein with its host molecular partners—ACE2, Furin and TMPRSS2 (not shown in the figure). The ACE2-S protein interaction initiates the endocytosis of viral assembly upon which the endocytic vesicle and viral membrane are dissolved and ssRNA together with nonstructural proteins (helicases, RNA-dependent RNA polymerase (RdRp), and 3C-like protease (3CLpro)) initiate the replication-transcription phenomenon. ER-Golgi complex initiates the translation, folding, packaging, and transport of viral proteins. Upon expression of viral RNA and proteins, the virus is assembled, packed into exocytic vesicle, and secreted out of the infected cell

Viral entry is the first interaction between the viral and human proteins. One of the common viral entry proteins is Spike glycoprotein (S-protein). The S-protein interacts with the ACE2 receptor and initiates the membrane fusion process with the help of host proteases such as TMPRSS2 and furin (Chang et al. 2021). Li et al. studied the ACE2 expression pattern in lung tissues of healthy and infected patients

Table 1.2 Examples of inhibitors and vaccines developed against SARS-CoV-2

Type	Name	Virus	Mechanism	References
Inhibitors				
Small molecule				
	SSAA09E2	CoV	Disturbing S-ACE2 interaction	Adedeji et al. (2013)
	Arbidol	CoV-2	Modulation of S protein trimerization	Vankadari (2020), Herod et al. (2019)
	Chloroquine	CoV-2	Atypical PPI inhibition	Wang et al. (2020)
	P3	CoV-2	Stabilizes non-native dimer of nucleocapsid protein	Lin et al. (2020)
Antibody/Antibody fragments				
	CB6	CoV-2		Shi et al. (2020)
	B38	CoV-2		Wu et al. (2020b)
	311mab-31B5 and 311mab32D4	CoV-2		Chen et al. (2020)
	COVA2-15	CoV-2		Brouwer et al. (2020)
	IgG1 ab1	CoV-2		Li et al. (2020b)
	ACE2-Ig	CoV-2		Lei et al. (2020)
Soluble peptide analogs of ACE2				
	hrsACE2	CoV-2		Monteil et al. (2020)
	S471-503	CoV	Disturbing S-ACE2 interaction	Hu et al. (2005)
	438YKYRYL443	CoV	Binding with host ACE2	Struck et al. (2012)
Peptide-based inhibitor				
	Octapeptide	CoV-2	Inhibitor of intradimer of 3CLpro	Wei et al. (2006), Gan et al. (2006)
	hexa-D-arginine amide (D6R)	CoV-2	Furin-mediated cleavage of S protein	Cheng et al. (2020)
Lipopeptide				
	EK1C4	CoV-2	Disturbing 6-HB formation of S protein	Cheng et al. (2020)
Vaccines				
Nonreplicating viral vectors				
	Jhonson & Jhonson vaccine	CoV-2		Shay (2021)
	Covishield	CoV-2		Jeewandara et al. (2021)
	Sputnik light vaccine	CoV-2		Komissarov et al. (2022)

(continued)

Table 1.2 (continued)

Type	Name	Virus	Mechanism	References
	Sputnik V vaccine	CoV-2		Jones and Roy (2021)
Protein subunit				
	Corbevax	CoV-2		Thuluva et al. (2022)
	Novavax	CoV-2		Mahase (2021)
RNA				
	Moderna vaccine	CoV-2		Wei et al. (2021)
	Pfizer-BioNTech COVID-19 Vaccine	CoV-2		Chagla (2021)
Inactivated virus				
	Covaxin	CoV-2		Sapkal et al. (2021)

by functional enrichment analysis and revealed the significant role of ACE2 receptors in lung infections. According to the study, ACE2 expression in infected tissues led to inflammatory response and cytokine storm by increased expression of proteins like SRC and CASP1. ACE2 was also reported to promote viral replication as expression of several viral hub proteins (RPS8, RPS8, RPS3) was found to be increased according to the PPI network analysis (Li et al. 2020a). Several small molecules and peptides have been developed as antagonists of Spike-Ace2 interactions (Table 1.2). For instance, Kalhor et al., identified diammonium glycyrrhizinate which is an FDA-approved drug as a potent inhibitor of viral entry via MD simulation technique (Kalhor et al. 2022). Similarly, isolated monoclonal antibodies have been identified as neutralizing agents of SARS-Cov-2 by interaction with S-protein or ACE2 (Wu et al. 2020b; Shi et al. 2020). The antibodies and ACE2 have been utilized for the development of soluble peptide analogs which can be used as potential treatment drugs. Montil et al., tested the efficacy of human recombinant ACE2 as a drug against SARS-Cov-2 in cell model. From the experiments, they found that the recombinant ACE2 reduced the viral replication by a factor of 1000 times (Monteil et al. 2020). Monoclonal antibodies fused with extracellular domain of ACE2 receptor have also been developed, which exhibited high affinity toward the receptor-binding domain (RBD) of Spike protein (Lei et al. 2020). In a similar fashion, the structural and molecular details of viral pathogenesis have been exploited in development of novel drugs against RdRP, nucleoprotein, membrane proteins and 3-CLpro (Table 1.2).

1.5.2 Drug Repurposing Strategy

Protein–protein interactions between host and SARS-Cov-2 are being extensively mapped with the help of high-throughput proteomic techniques, bioinformatic tools and databases. For instance, Gordon et al., identified 332 PPIs involved in SARS-Cov-2 infection by affinity purification-mass spectrometry (AP-MS) (Gordon et al. 2020). Further the viral mechanisms were unraveled by comparative study of several host-coronavirus PINs. These PINs helped in drug repurposing and designing of PPI modulators. The repurposing strategy is highly dependent on holistic knowledge of molecular networks (Kumar 2021). This strategy is cost-efficient and more streamlined as compared to traditional drug design methodologies thus making it a desired approach (Adhami et al. 2021).

Several methods such as gene expression data and proteomics have been applied to curate the information of molecular networks involved in a disease symptom. The network study allows association of SARS-Cov-2 with host PPI networks and attempted to model or reutilize known drugs for treatment of the COVID-19 infections. Zhou et al. utilized an antiviral drug reengineering approach to measure the association of SARS-Cov-2 with known antiviral medicines (Zhou et al. 2020). Using the network analysis, 16 drugs were identified as broad-spectrum antiviral drugs against COVID-19 infections. Similarly, Gysi et al. have discovered 208 human proteins that can be targeted by SARS-Cov-2 (Gysi et al. 2021). Further, a special online data analysis tool CoVex has been developed that incorporates SARS-Cov strains-human PINs interaction for identification of novel and reusable drugs (Sadegh et al. 2020). Similarly, VirHostNet is a database that stores information of manually annotated PPIs from various coronaviruses (Messina et al. 2020). A list of databases and tools such as CoVex (Chukwudozie et al. 2021), VirHostNet, CORDITE, P-HIPSTer (Martin et al. 2020; Singh 2019) have been recently developed using viral-host PPIs. Moreover, novel bioinformatic algorithms and models can also help in investigation of PPIs involved in COVID-19 infections. Other than discovery of drug targets, these algorithms and models can be useful in identification of stages of infection such that an accurate treatment protocol can be devised. Khorsand et al., developed a novel PPI prediction model for accurate identification of SARS-Cov-2-human PPIs using a three-layer network. The initial layer consists of viral proteins like SARS-Cov-2 proteins; the second layer contains PPIs between SARS-Cov-2-related viral proteins, and host proteins; and the last layer consists of PPIs specifically for SARS-Cov-2 and human proteins. From the network analysis, Khorshand et al., predicted 7201 interactions between 11 viral proteins and 1898 human proteins. They also predicted the key residues and motifs utilized/targeted by SARS-Cov-2 for causing infection in humans (Khorsand et al. 2020).

1.6 Intrinsic Disorder-Based Human Diseases

Intrinsically disordered proteins (IDPs) are native proteins which do not contain stable secondary or tertiary structures. They are abundantly available in cells, involved in signaling and regulation of cellular processes. IDPs and intrinsically disordered regions (IDRs) generally undergo post-translational modifications (Uversky 2015). IDPs/IDRs are associated with various diseases such as cancer, diabetes, cardiovascular diseases, and neurodegenerative diseases (Choudhary et al. 2022; Coskuner-Weber et al. 2022). IDPs lack three-dimensional structure either entirely or in parts. The disordered regions have several inherent characteristics such as conformational flexibility, increased surface area for protein–protein interactions, and molecular recognition features. They can easily form a scaffold and interact with proteins moreover their irregular structure facilitates post-translational modifications for the proper regulation and function of a protein in the cell. Moreover, the disordered structure allows IDPs to interact with the target proteins with utmost specificity, although their affinity of binding is low. In several signaling molecules and receptors, the interacting interface has been characterized as IDRs. In addition to this, IDRs also mediate protein packaging and trafficking phenomena during translational and post-translational processes (Yang et al. 2021; Hosoya and Ohkanda 2021). As unstructured model promotes multiple binding partners of IDPs, their physiological concentrations need to be kept under check. Important signaling proteins such as SH2, PTB, and PDZ domain-containing proteins are known to be IDR-containing proteins (Fonin et al. 2019; Bondos et al. 2022). In addition to the latter, proteins involved in protein folding and transport such as scaffolding proteins also consist of IDRs to provide conformational flexibility.

The concentration and availability of IDPs affect the homeostasis of the cell environment as several diseases such as cancer and neurodegenerative disorders are associated with their altered availability (Ayyadevara et al. 2022; Mészáros et al. 2021). For instance, overexpression of stathmin, tau, and huntingtin proteins are associated with pathological conditions such as cancer and neuronal degeneration (Cusan et al. 2018; Birol and Melo 2020). Similarly, underexpression of IDPs have also been associated with cancerous pathological states. The regulation of IDPs is a crucial process for normal cellular functions. Numerous studies have analyzed the regulation of IDPs in the cell. The regulation of IDPs in the cell is governed by three principles. First, the IDPs are regulated at transcript level as well as at protein degradation. The synthesis and availability of IDPs are regulated through multiple mechanisms thereby enhancing the fidelity and minimizing the risk of nonfunctional, inappropriate interactions during their short lifespan. The second principle states that dosage-sensitive genes such as oncogenes are rich in IDPs, and such genes express proteins containing linear peptide and are tightly regulated at mRNA and protein level (Vavouri et al. 2009). Third, availability of IDPs is fine-tuned according to cell requirements. Stress conditions and various cell-cycle phases promote the expression of IDPs despite the tight regulation of their transcription and translation, thus increasing the concentration and accessibility of IDPs.

As IDPs mostly have a short half-life, several nanny proteins are present in the cell to enhance the half-life of IDPs. These nanny proteins inhibit the proteasomal degradation process by associating with the IDPs to increase their lifespan. This principle has been investigated and established in the case of tumor-suppressing protein p53, and its paralog p73 (Åberg et al. 2018; Neira et al. 2021). Post-translational modification also plays an important role in maintaining the levels of IDPs in the cell. PTMs, especially phosphorylation can alter the stability of IDPs, and fine tune their concentration (Owen and Shewmaker 2019; Acosta et al. 2022). Further, IDPs engagement in protein interaction networks such as cell signaling cascades is concentration-dependent and multitiered. For instance, GSK3 is a signaling factor that regulates Wnt as well as insulin-signaling pathway. Similarly, Oct4, Sox2, SH2 domains are involved in activation of MAP kinases and MAPK pathways. To avoid crosstalk and inappropriate outcomes, the cell ensures a spatio-temporal accumulation of IDPs and signaling complexity, thus encouraging their discrete activity in the signaling cascades. Other than signaling proteins, transcription factors (TFs) are also intrinsically disordered in nature, and such unique characteristic of TFs aids in formation of a highly specific interactome (Tsafou et al. 2018).

Owing to the relevance of IDPs in cellular signaling, they are considered to be the potential drug targets for various diseases. A functional enrichment study conducted for annotation of IDPs and IDRs function has revealed that IDPs are essentially associated with cancers and other related malignancies (Deiana et al. 2019). Small molecules and short IDP mimicking/inhibiting peptides have been developed to either interact with the IDPs or the IDP-interacting interface of their binding proteins. For example, compounds which interact with the IDR region of Myc protein have been developed to inhibit Myc-Max complex formation in case of tumor cells (Singh et al. 2022). Similarly, peptide inhibitors of PDZ domain of disheveled protein have been designed which inhibit cellular differentiation and growth for tumor cells in several cancers (Gutiérrez-González et al. 2021). Fusion proteins formed due to chromosomal translocation and fusion of two coding genes are very common in several sarcomas and leukemia. The fusion of transcription factors in general results in the formation of IDPs. Fusion proteins such as EWS-FL1 and AF4-AF9 are common diagnostic and druggable targets in case of Ewing sarcoma and mixed lineage leukemia respectively (Santofimia-Castaño et al. 2020). IDPs are also targeted by disrupting the key IDP modulatory enzymes using small-molecule inhibitors to regulate their bioavailability and stability. Puca et al. analyzed the inhibition properties for a variety of secondary metabolites against Sirt1-deacetylase, which reduce the availability of HIPK2 kinase and increase the stability of p53 protein leading to apoptosis of cancer cells (Wang et al. 2018; Puca et al. 2010). Similarly, inhibitors of tau deglycosylase and phosphorylase in Alzheimer's disease have been identified for treatment of the same (Uversky 2015; Babu et al. 2011). Table 1.3 describes various diseases related to IDPs and IDRs (Tsafou et al. 2018; Uversky 2015).

Table 1.3 Examples of IDPs and IDRs containing proteins and their associated disease

Protein (IDP/IDR)	Diseases caused
Aβ	Alzheimer's disease; Dutch hereditary cerebral hemorrhage with amyloidosis; congophilic angiopathy
Tau	Tauopathies; Alzheimer's disease; corticobasal degeneration Pick's disease; progressive supranuclear palsy
Prion protein	Creutzfeldt-Jacob disease; Gerstman-Sträussler-Schneiker syndrome; Fatal familial insomnia; Kuru; Bovine spongiform encephalopathy; Scrapie
FUS	Amyotrophic lateral sclerosis
Ataxin-2	Spinocerebellar ataxia2
Huntingtin	Huntington's disease
p53, c-Myc	Multiple types of cancer
Sp1	Alzheimer's disease; multiple types of cancer
TAF4	Ovarian cancer
KLF5	Cardiovascular diseases

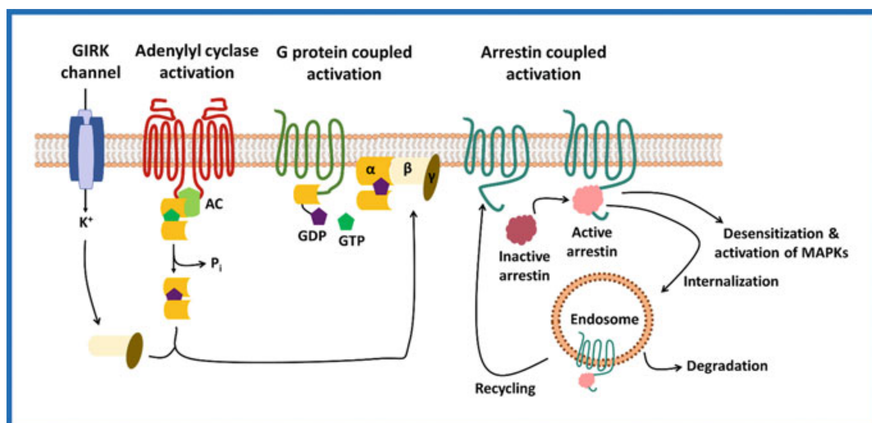

Fig. 1.2 Illustrative schematic depicting various GPCR-associated signaling pathways and their immediate downstream effector molecules. *GIRK,* G-protein-gated inwardly rectifying potassium channels; *AC,* adenylyl cyclase; *GDP,* guanosine-5′-diphosphate; *GTP,* guanosine-5′-triphosphate; *MAPK,* mitogen-activated protein kinase

1.7 PPIs in GPCR-Related Diseases

GPCRs or G-protein coupled receptors are ubiquitous transmembrane proteins which consist of a single polypeptide chain with seven transmembrane domains (Bohme and Beck-Sickinger 2009). GPCRs are crucial for physiological functions, and mediate cell signaling via various ligand molecules (Fig. 1.2). GPCRs are divided into five major families based on sequence and structural similarities. The five families are rhodopsin, secretin, glutamate, adhesion and frizzled/Taste2. All

five GPCR families share structural similarities even though they are involved in unique signal transduction activities. GPCRs mediate signal transduction by creation of a signaling cascade, which is either dependent or independent of intracellular G-proteins. In both the cases, activation of several downstream effectors such as adenylyl cyclase, tyrosine kinases, and phospholipases are involved in stimulating the relevant downstream cellular functions (Hilger et al. 2018). The functional diversity of GPCRs has been reviewed elsewhere (Borroto-Escuela and Fuxe 2019; Milligan and White 2001). GPCRs are involved in various human diseases such as neurodegenerative disease (Moreno et al. 2009; Fuxe et al. 2014), HIV, cancer (Lappano and Maggiolini 2011), hypertension (Brinks and Eckhart 2010; Clark et al. 2021), etc. Owing to this, GPCRs are a potent druggable target for pharmaceutical compounds (Hauser et al. 2017; Wacker et al. 2017; Congreve et al. 2020). Till date, over 134 GPCRs are approved as drug targets by FDA and ~700 drugs, i.e., 35% of approved drugs are used to target GPCRs in diseases (Sriram and Insel 2018). Several techniques such as coIP, AP-MS, protein microarray have been utilized for characterization of GPCR-interaction with protein complexes (Daulat et al. 2009; Chung et al. 2013; Benleulmi-Chaachoua et al. 2016; Poluri et al. 2021a). Yeast two-hybrid systems have also been used for detection of PPIs involving disease-relevant GPCRs. A study conducted in 2017 defined the interactomes of 48 diseases with relevant GPCRs that play essential role in disease prognosis and progression (Sokolina et al. 2017).

Neurodegenerative diseases such as Alzheimer's is one of the prime examples of GPCR-related diseases. A plethora of research is available on the implications of GPCRs in pathogenesis of Alzheimer's disease (AD). AD involves processing and accumulation of amyloid proteins which lead to neurodegeneration. GPCRs are involved in multiple stages of amyloid precursors. Zhao et al. reviewed the function of β-secretase (BACE1)-related GPCRs that are actively involved in processing and further accumulation of amyloids in the brain (Zhao et al. 2016). Similarly, microglial GPCRs have also been implied in protective as well as detrimental effects in the case of Alzheimer's disease. Microglial GPCRs are also involved in the processing of beta amyloid proteins in addition to their role as regulatory proteins mediating beta amyloid degradation, phagocytosis, and chemotaxis (Haque et al. 2018). Acetylcholinesterase, a synaptic enzyme is one of major GPCR-binding proteins, and is considered as a potential druggable target for treatment of Alzheimer's disease (Gao et al. 2016b, 2021). Likewise, GPCRs are primarily targeted in cases of cancer, as tumor cells tend to hijack and overexpress GPCRs for increasing the tumor cell growth exponentially (Usman et al. 2020). Several anticancer drugs have been developed which target GPCR-related protein–protein interactions. For example, chemokines such as CXCL8 which binds to CXCR1 and CXCR2 are targeted for treatment of melanoma, pancreatic cancer, and gastric cancers (Liu et al. 2016). Similarly, secretion and interaction of exocrine hormones are targeted for the treatment of prostate cancer (Baratto et al. 2018; Crawford et al. 2018). The role of GPCR protein–protein interactions in diseases has been explained elaborately in the following chapters. An overview of GPCR-targeting drugs and their mechanistic action for treatment of diseases is mentioned in Table 1.4.

Table 1.4 Summary of GPCR-targeting drugs in diseases and their mechanism of action

Mechanism of action	Target receptor	Drug	Disease
Indirect orthosteric agonism or antagonism by changing natural ligand concentration	DPP4 (GLP-1, GIP receptors)	Sitagliptin (Dhillon 2010)	T2DM
	ACE (AT1, AT2 receptors)	Captopril (Leier et al. 1983)	Hypertension
	Cyclooxygenase (many PG, lipoxin, and Tx receptors)	Aspirin (Desborough and Keeling 2017)	Pain and inflammation
Indirect antagonism by reducing receptor expression	Glucagon receptor	ISIS 325568 (Phase II) (van Dongen et al. 2015)	T2DM
Negative allosteric modulation	CCR5	Maraviroc (Perry 2010)	HIV/AIDS
Positive allosteric modulation	CaS receptors	Cinacalcet (Balfour and Scott 2005)	Secondary hyperparathyroidism
Orthosteric antagonist	β1- and β2-adrenoceptors	Propranolol (Al-Majed et al. 2017)	Cardiac arrythmia and heart failure
	H2 receptors	Ranitidine (Dave et al. 2004)	Dyspepsia
	GnRH receptors	Degarelix (Frampton and Lyseng-Williamson 2009)	Prostate cancer
	Somatostatin receptor	Lanreotide (Burness 2015)	Pancreatic cancer
	CXCR4	Plerixafor (DiPersio et al. 2009)	Multiple myeloma
	Smoothened receptor (SMO)	Sonidegib (Burness 2015)	Locally advanced and metastatic basal cell carcinoma
	CCR4	Mogamulizumab (Subramaniam et al. 2012)	T cell lymphoma
Orthosteric agonist	β2-adrenoceptors	Salbutamol (Cullum et al. 1969)	Asthma
	GLP-1 receptors	Exenatide (Cvetković and Plosker 2007)	T2DM
	SST2 and SST5 receptors	Octreotide (Lamberts and Hofland 2019)	GH-secreting tumors and acromegaly
	Dopamine receptor D1	Cabergoline (Colao et al. 2000)	Neuroendocrine tumors, pituitary tumors

1.8 PPIs Related to Cataract Formation

Cataract is opacification of the eye lens leading to temporary or permanent loss of eyesight. The eye lens is normally a biconvex structure composed of fibers, surrounded by thin capsules and zonules on both sides. The fibers are made up of epithelial cells and move inwards to the center from the periphery. Therefore, the nucleus contains older fibers, whereas the newly formed fibers are present at the periphery (Nartey 2017). The fibers express a structural protein called crystallin that is responsible for optical properties of the lens (Roskamp et al. 2020a, b). The proper functioning of crystallin protein depends upon hydration and their native configuration. Hydration and ionic equilibrium are maintained across the lens by membrane proteins, and the cytoskeleton maintains the shape of fiber cells. The sulfhydryl group of soluble crystallin remains in reduced state under normal conditions due to the presence of reduced antioxidants such as glutathione. Under stress conditions such as aging, the concentration of reactive oxygen species (ROS) goes up and causes oxidative stress. The stress condition creates alterations in the redox state of the cells, promoting disulfide formation and oligomerization of crystallin resulting in precipitation of crystallin and opacity of lens (Fig. 1.3a) (Nartey 2017). The precipitation is further affected by absence of glutathione in the nucleus region and lifestyle disorders such as diabetes mellitus. Based upon the causative factors, cataracts are classified into three categories: age-related cataracts, cataractogenesis (cataracts secondary to other causes) and pediatric cataracts (Liu et al. 2017).

One of the main causes of cataracts worldwide is aging. The onset of cataract generally happens between the age of 45 and 50 years in adults. The opacification can take place at different areas within the lens; based upon the localization of aggregation/opacification, age-related cataract is divided into three types: nuclear, cortical and posterior subcapsular cataracts (Liu et al. 2017). In nuclear cataract, the fiber cells migrate to the central portion of the lens and undergo ROS-mediated oxidation, insolubilization and crosslinking, thus resulting in nuclear sclerosis and opacity (Fig. 1.3b). While in cortical cataract, the aggregation is often wedge-shaped and starts at the cortex, later spreading to the nucleus (Fig. 1.3c). Further, the subcapsular cataract is localized in the axial posterior curtail layer and the opacity is plaque-like in appearance (Fig. 1.3d) (Michael and Bron 2011). The age-related accumulation of PTMs by glycation of proteome is one of the major mechanisms of cataract (Fan and Monnier 2021; Cantrell and Schey 2021). Pediatric cataracts occur in infants and are the leading causes of childhood blindness. Based upon the time of its occurrence, pediatric cataract is of two types, congenital and infantile cataract. Congenital cataract is when opacity is present at birth, while infantile cataracts refer to the development of lens opacity during the first year of birth. Pediatric cataracts are mostly inherited or associated with sporadic ocular anomalies and congenital diseases (Gasper et al. 2016; Khokhar et al. 2017). In addition to internal factors, cataract is also induced by environmental or foreign factors. External factor-induced cataract is known as cataractogenesis (Andjelić and Hawlina 2012). The most common cause of cataractogenesis is usage of corticosteroids (James 2007). All factors involved in induction of cataract are mentioned in Table 1.5.

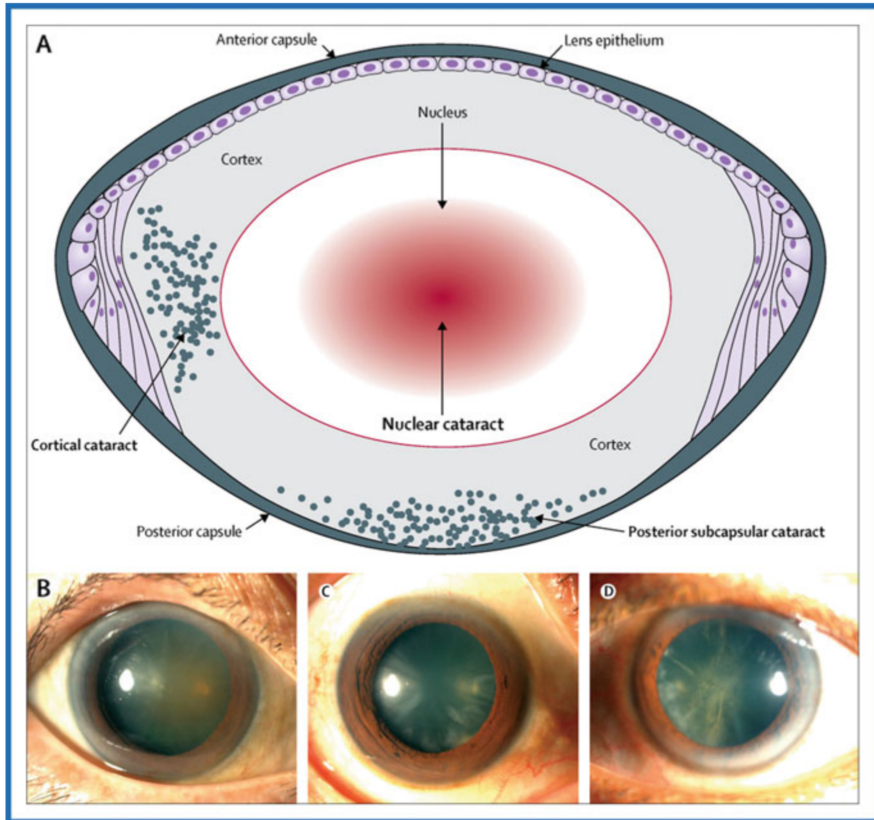

Fig. 1.3 (**a**) Illustrative figure depicting different types of cataracts based upon the localization of crystallin precipitation. Real-time images showing the differential structural changes in (**b**) nuclear cataract, (**c**) cortical cataract, and (**d**) subcapsular cataract. (Adapted from Liu et al. 2017)

Table 1.5 Common causative factors of various types of cataracts (Nartey 2017)

Type of cataract	Causes
Age-related cataracts	Increasing age
	Type 2 diabetes
	High blood pressure
Pediatric cataract	Idiopathic
	Ocular anomaly
	Down's syndrome
	Hypoglycemia
	Trisomy 13–15 syndrome
	Myotonic dystrophy
	Maternal infections
Cataractogenesis	Corticosteroids
	Ultraviolet-B exposure
	Cigarette smoking

1.8.1 Etiology of Cataract

The understanding of cataract pathophysiology has gained a lot of traction over the past decade. Due to the diversity in cataract morphologies, the accurate biochemical process behind each morphology has not yet been resolved. However, crystallin is the main protein involved in all cataract morphologies despite the varied causal factors. Crystallins are water-soluble lens proteins divided into two families called α-crystallin and βγ-crystallin. They constitute about 90% of the lens protein, and are accountable for the refractive and transparent properties of the lens (Moreau and King 2012). α-crystallins are also molecular chaperones that maintain the integrity of the cytoskeleton. Other than lens, α-crystallins are expressed in heart, skeletal tissue, brain, and other tissues as well (Moreau and King 2012). During cataract, crystallin lose their soluble property and conformational stability to cluster together forming an opaque sheath on the lens (Roskamp et al. 2020b). These changes are initiated by a variety of factors, of which, aging and genetic mutations are the leading causes of cataracts. Mutations in α-, β-, and γ-crystallin are mainly responsible for the early onset of cataract. Many mutations such as R14C, P23T, R36S, W156, and R58H in γD-crystallin have been extensively characterized (Fu and Liang 2003; Ghosh and Chauhan 2019). Increased hydrophobicity due to mutations also increases the propensity of aggregation as shown by NMR studies on G18V γS-crystallin variant (Khago et al. 2016). Moreover, these mutations are also known for reducing the stability of crystallin–crystallin interactions. Other γ-crystallin, α-crystallin, and β-crystallin mutations have also been accounted for their detrimental effect on the lens (Song et al. 2020).

Beside genetic mutations, ROS-inducing factors (aging, UV radiation, heavy metals, steroid treatments), post-translational modifications (deamidation, oxidation, glycation), and lifestyle-associated diseases (diabetes and asthma) can also cause cataracts. ROS-inducing factors also influence the onset of cataract in patients as the lens environment is rich in glutathione, and low levels of oxygen protect the lens proteins from oxidation. With age, the concentration of glutathione decreases in the lens and contributes to development of cataract. The increase in oxidative molecules has been linked to dysregulation of calcium content and activation of calpains which promote progression of cataract (Vu et al. 2022). UV radiations are also involved in generation of ROS and affect the stability of crystallin (Moreau and King 2012). Post-translational modifications also affect the stability of crystallin proteins. One of the examples of PTM-mediated changes in crystallin is glycation. Diabetes-associated cataracts are a result of glycated crystallin. The high concentration of glucose in diabetic patients leads to protein glycation affecting the chaperone activity of crystallin and thus alters the tonicity of the lens (Zhu et al. 2019). Deamidation is also one of the prevalent causes of crystallin aggregation. Proteome analysis has revealed that deamidation introduces negative charge to the protein by Asn → Asp or Gln → Glu transformation (Truscott and Friedrich 2016). Other than crystallin, a variety of structural proteins are also involved in development and progression of cataract (Fig. 1.4). The associated genes are used as biomarkers for detection of inherited cataract (Zhu et al. 2017).

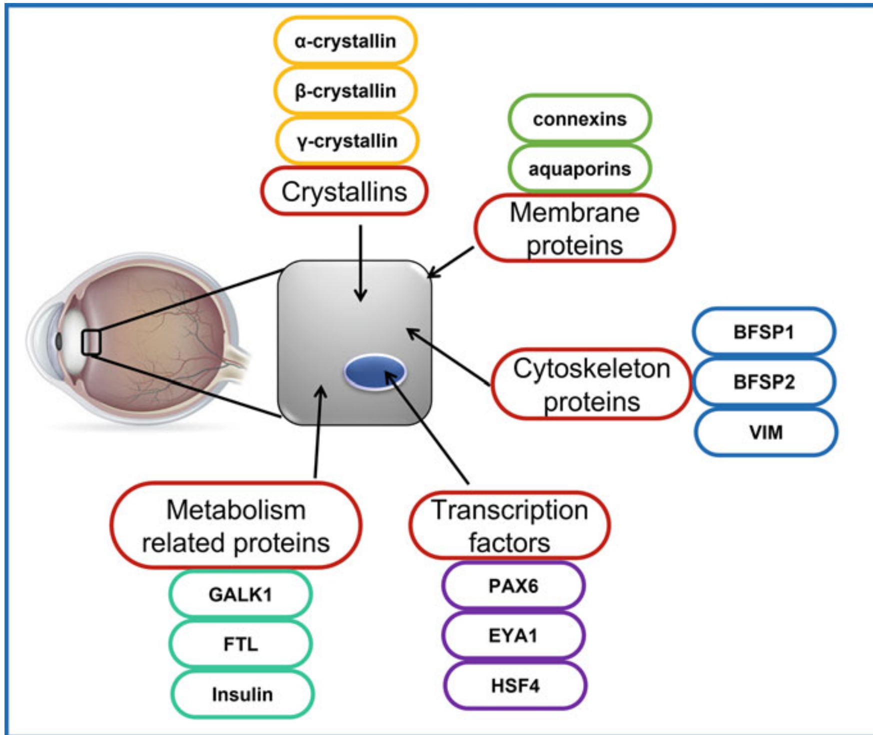

Fig. 1.4 Schematic illustrating the five major protein families including crystallins, membrane protein channels, cytoskeleton proteins, transcription factors, and metabolism-associated protein that partake in the etiology of cataract formation together with their respective examples

Besides crystallins, structural alterations in membrane proteins are also linked to cataracts. Lens is an avascular organ, hence, to perform its functions, a proper hydration needs to be maintained. Connexins are transmembrane domain proteins which allow intercellular communication. Mutations in connexins have been associated with diverse human diseases such as deafness, skin diseases, cardiovascular diseases, and cataract. In the healthy eye lens, connexins circulate ions and water to maintain the homeostasis and transparency. Further the C-terminal of connexins interacts with several lens proteins such as crystallin, major intrinsic protein (MIP), etc., to maintain vascular environment. Recently, Minogue et al., showed the relevance of serine mutations in connexin50 via cell culture and immunoblotting studies. Absence of serine connexin50 led to absence of phosphorylation, which is crucial for functioning and protein–protein interactions. The lack of phosphorylation led to exposure of a sorting signal that promotes the lysosomal degradation of connexin50 (Minogue et al. 2022). Similarly, MIP also acts as a water channel in the lens and interacts with crystallins and other regulatory lens proteins (Hejtmancik et al. 2015; Sun et al. 2021). Cataract is also associated with several eye diseases for instance, myopia is associated with nuclear cataract and posterior

subcapsular cataract (Ang and Afshari 2021). In such conditions cataract is a result of dysregulation of protein–protein interactions involving all ocular compartments. Ocular compartments such as aqueous humor (AH) and vitreous humor (VH) are responsible for providing nutrition and removing waste from the avascular compartments of the eye. Eye disorders trigger spatiotemporal changes in expression and interactions of proteins in AH and VH and stimulate cataract formation (Joachim et al. 2007). A study conducted in 2015, reported 77 AH proteins to be associated with cataract in myopia, glaucoma, and vitrectomy surgery patients. Out of the 77 proteins, 17 proteins were found to be involved in PPI networks, while five proteins were directly associated with cataract and nervous system diseases (Ji et al. 2015).

1.9 PPIs Involved in Cystic Fibrosis

Cystic fibrosis is a heterozygous recessive genetic disorder where cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations affect the production of mucus in different organs of human body. The CFTR gene is responsible for the expression of transmembrane channels, involved in transport of chloride ions across apical membranes of epithelial cells of pancreas, lungs, sweat gland, and male reproductive tract (Knowles and Durie 2002). It is also responsible for inhibition of sodium channels (ENaC) involved in hydration and secretion of mucins (Fig. 1.5). The mutations in CFTR lead to chronic respiratory infections, deficiency in pancreatic hormone, partial breakdown of fats (maldigestion), obstructive azoospermia, and hypersecretion of chloride in sweat. While classic cystic fibrosis accounts for double loss of function mutations, nonclassical cystic fibrosis accounts for single copy of mutant gene conferring partial function of CFTR protein. Based upon the kind of defect in CFTR, mutations are divided into six classes (Table 1.6) (Bell et al. 2020).

Of all CFTR mutations, F508DEL (Class II) is the most prevalent cause of cystic fibrosis (Pankow et al. 2015). CFTRs are members of ATP-binding cassette membrane transporter family. Wild type isoform of CFTR comprises two multipass membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory domain. The regulatory domain interlinks an MSD to NBD. The F508DEL mutation is localized in the NBD1 region and modifies the local conformation of the NBD1 domain. The increased disorderness in the NBD1 regions disrupts interaction between NBD1 and intracellular loop 4 (ICL4) of MSD2 domain leading to misfolding of CFTR protein. The misfolded protein undergoes the proteasome led degradation, leading to absence of functional CFTR and low anion trafficking (Fig. 1.5) (Wang and Li 2014). Mutations-driven effects on CFTR differ in their phenotype and the intensity of diseased state. Class I, II, and III mutations generally result in either no expression of mRNA/protein or no trafficking/gating leading to severe diseased state (Stanke and Tümmler 2016). Class IV, V, and VI mutations impair the functions of CFTR protein, reducing the rate of anion transport or stability of the CFTR protein. The latter is mainly related to less-severe diseased

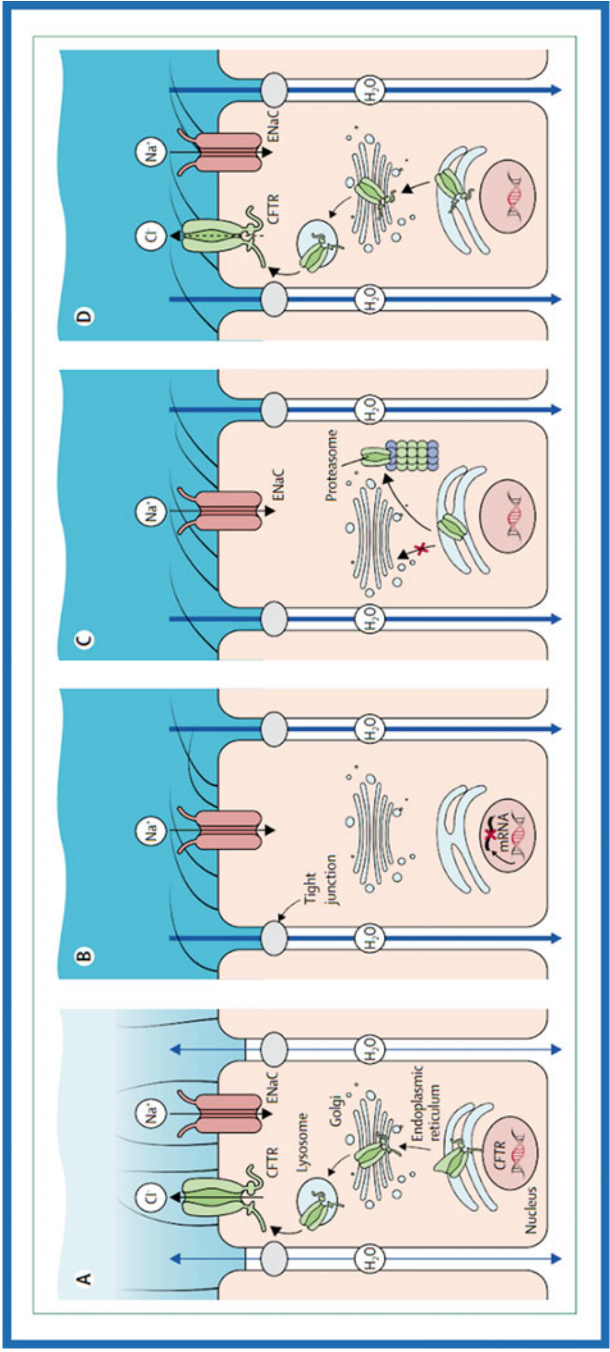

Fig. 1.5 Schematic depicting the pathophysiology of cystic fibrosis. (**a**) In a healthy individual, wild type CFTR is expressed at the apical surface epithelial cells. CFTR and ENaC work in a coordinated manner for effective mucociliary clearance and hydration. However, in case of cystic fibrosis mutations, the CFTR becomes nonfunctional (**b**) or dysfunctional (**c** and **d**) causing disruption of mucociliary clearance pathway and extracellular trafficking of chloride ions. (Adapted from Bell et al. 2020)

Table 1.6 CFTR classification and their examples (Rowntree and Harris 2003)

CFTR class	CFTR defect	Type of mutation	Examples
CFTR Class I	Functional CFTR protein absent	Nonsense; canonical splice; frameshift	G542X; W128X; R553X; 621+1G→T
CFTR Class II	CFTR trafficking defect	Missense; amino acid deletion	F580DEL; N1303K; I507DEL; R560T
CFTR Class III	Malfunction of channel regulation	Missense; amino acid deletion	G551D; G178R; G155S; S549N
CFTR Class IV	Reduced channel conductance	Missense; amino acid deletion	R117H; R347P; R117C; R334W
CFTR Class V	Lowered synthesis of CFTR	Splicing defect; missense	3849+10kbC→T; 2789+5G→A; 3120+1G→A; 5T
CFTR Class VI	Lower CFTR stability	Missense; amino acid change	432delTC; Q1412X; 4279insA

states, and in general affects a single organ (Table 1.6). The phenotypic effects are a result of impaired protein–protein interactions involving the CFTR protein (Rowntree and Harris 2003).

Class I mutations occur due to nonsense or frameshift mutations leading to premature termination of protein synthesis. The truncated protein is recognized by chaperones and is degraded. The most common class I mutations are G542X, W128X, and R553X. While compared to class I mutation, ***Class II*** mutations are resultant of missense mutation or amino acid deletions which affect the protein maturation process. Even though a full-length mRNA is transcribed, such mutations lead to translation of nonfunctional CFTR proteins. Some examples of class II mutations are F508DEL, N1303K, I507DEL, and R560T. While class I and II lead to no protein expression, ***Class III*** mutations affect the regulation and gating of the anion channel as CFTRs are ABC transporters and are stimulated via cAMP for the chloride ion trafficking. Class III mutations occur in the nucleotide-binding region and the regulatory domain thus preventing the conformational change and affecting the ATP binding. Examples of class III mutations are G551D, G176R, G551S, and S549N. The implications of the rest categories of mutations are less severe. Class IV, V, and VI mutations are either missense mutations or splicing defects (Fig. 1.6). These defects end up in reduced channel conductance, synthesis, and stability of CFTRs respectively. Some examples of such mutations are mentioned in Table 1.6.

The involvement of CFTRs in chloride ion trafficking is incomplete without Na^+/K^+ ions transporters. In a normal healthy cell, anion and cation transporters work hand in hand to maintain the ionic balance and pH of the cellular milieu. Defected CFTRs hinder anion transport together with affecting the transport of sodium and potassium ions. Two cation channels, ENaC and KCa3.1 are directly regulated by CFTR protein. The protein–protein interactions between ENaC and CFTRs are widely known, while CFTR-KCa3.1 interactions in the airway have been proven much recently (Klein et al. 2016). KCa3.1 maintains the K^+ efflux at the apical and

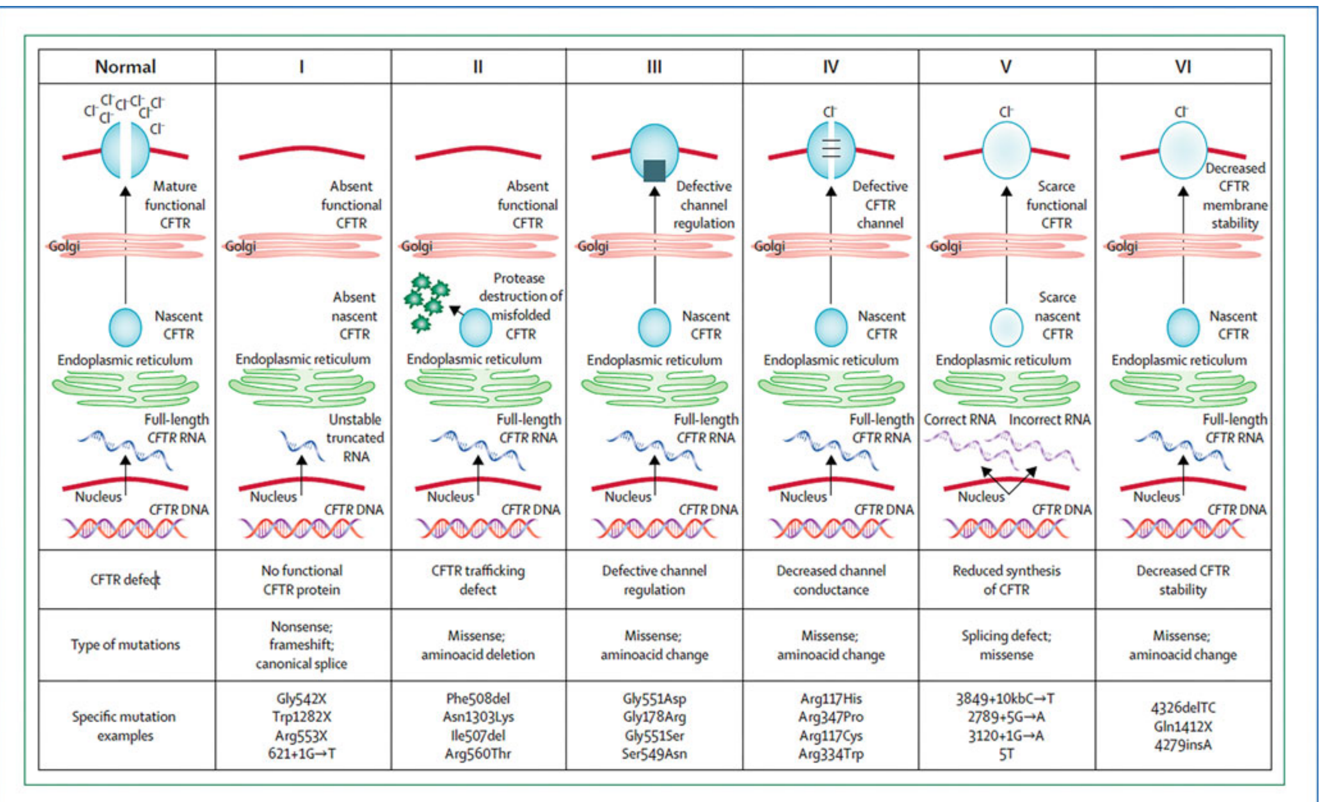

Fig. 1.6 Schematic representation of different classes of CFTR mutations. The CFTR gene mutations are categorized into six classes. Class I mutations result in nonfunctional CFTR protein while Class II and Class III mutations relay defect in CFTR trafficking and channel regulation respectively. The latter mutations—IV, V, and VI result in formation of dysfunctional CFTR protein which shows decreased channel conductance, CFTR synthesis, and CFTR stability respectively. (Adapted from Bell et al. 2020)

basolateral membrane during transepithelial transport of Cl^- ions by CFTRs. Two-hybrid screen experiments have been used to decipher the physical interaction between CFTR and KCa3.1. The experiment elucidated the intracellular Ca^{2+} ion-dependent association of N-terminal and calmodulin-binding region of KCa3.1 with NBD2 and C-terminal region of CFTR (Klein et al. 2016).

Other than cation channels, a wide variety of cellular proteins regulate CFTRs at the time of synthesis, folding, trafficking, and conductance. The CFTR interactome is referred to as the CFTR functional landscape. The interaction partners are connected either to the proteostasis network (CFTR interactome and secretome) or CFTR function (CFTR functionome) (Amaral et al. 2020). Identification of CFTR functional landscape is aimed to help with introduction of systemic options of diagnosis, prognosis, and treatment for cystic fibrosis patients. Characterization of interaction partners has helped in identifying potential CFTR correctors for some of the most common mutations. These correctors contribute to rectifying the defective trafficking, and CFTR functioning. As mentioned earlier, defective trafficking is a result of class II mutations. AP-MS technique has been employed to study the protein interaction partners (PIP) of four class II variants namely, F580DEL, R560T, G85E, and N1303K (Amaral et al. 2020). All these four variants share similar PIPs and downregulate the trafficking efficiency of CFTR variants. siRNA-mediated silencing of class II variants has also shown improvement in the trafficking efficiency. One of the common correctors, lumacaftor has also been revealed to convert the F580DEL PIP to a wild type-like interactome (Boyle et al. 2014; Deeks 2016).

F580DEL variants are easily recognized by the ubiquitin-proteasome pathway in the endoplasmic reticulum (ER). Functional genomics approach has been utilized for characterization of proteins involved in inhibition of F580DEL trafficking. More than 200 genes have been identified to be involved in the F580DEL trafficking (Amaral et al. 2020). Gene silencing methods were applied for such genes, and it was found that gene silencing rescued the F580DEL variant from degradation. Similarly, several correctors have been developed to mask the F580DEL variant in such a way that the proteasomal degradation is inhibited (Devesa et al. 2013). Similarly, the CFTR functionome has been identified using a combination of siRNA screening approach and halide-sensitive yellow fluorescent protein-mediated monitoring of CFTR-secretome interactions. Several key proteins such as RNF5/RMA1, E3-ubiquitin ligase, UBA2 (Ub ligase in sumoylation pathway), and UBXD1 (involved in ER-associated degradation) are pivotal targets for attenuation of cystic fibrosis and development of CFTR correctors (Nagahama et al. 2009; Ahner et al. 2013; Sondo et al. 2017). A fusion protein, FAU made up of FUB1 and ribosomal protein (30S) is also responsible for CFTR variant degradation, and proven as a relevant therapeutic target (Tomati et al. 2019). Syntaxins are another group of proteins that regulate the function of CFTRs. Syntaxins interact with the amino terminal of CFTR and inhibit its processing (Naren et al. 1998; Arora et al. 2021; Sabirzhanova et al. 2018; Csanády et al. 2019).

1.10 PPIs in Ubiquitin-Proteasome Pathway

The ubiquitin proteasome pathway (UPP) is a protein destruction pathway that controls the functional activities of different proteins. The ubiquitin-proteasome pathway recognizes misfolded/non-native proteins with the help of ubiquitin, which is recognized by the proteasomal unit to degrade the protein. The UPP is an ATP-dependent pathway that utilizes the 26S proteasomes. The misfolded proteins are tagged with ubiquitin via ubiquitin ligases which then acts as a guiding map for the proteasomal degradation of the protein. Ubiquitin (Ub) is a small protein composed of 76 amino acid residues. It is bound to the target protein by an isopeptide linkage between the C-terminal glycine of ubiquitin (Pickart and Eddins 2004). Ubiquitins form a polyubiquitin chain by covalently interacting with the ε-NH_2 group of lysine present on the Ub surface (Kravtsova-Ivantsiv and Ciechanover 2012). The polyubiquitination is achieved by enzymes known as E1, E2, and E3 which activate, transfer, and ligate the Ub to the target protein. The 26S proteasome system specifically recognizes the Lys48-based polyUb chains and degrades the protein (Kravtsova-Ivantsiv and Ciechanover 2012). However, various other degrading signals are recognized by the UPP as well. This provides selectivity and specificity to the UPP system. The 26S proteasome is a large polymeric protease which acts as a chamber for protein degradation (Dahlmann 2016). Once the protein is inside the proteasome, polypeptides are digested into shorter peptides of 2–10 residue in length. The whole unit is made up of a 20S core particle (CP) and a 19S regulatory particle (RP) (Bard et al. 2018). The 20S CP looks like a barrel and is responsible for the catalytic activity composed of four stacked rings (two outer α rings and two inner β rings). The rings are further composed of seven distinct subunits. The 19S RP acts as base and lid to the barrel structure and recognizes ubiquitinated proteins. The RP is composed of 17 subunits (nine in the base complex and eight in the lid complex) (Bard et al. 2018) (Fig. 1.7).

The UPP performs a major role in regulation of cell cycle, cancer and cell survival, inflammatory response, and immune response. Proteins such as SCF, Mdm2 are different types of E3 ligases responsible for regulation of S phase during cell cycle and DNA repair (Paul 2008). The ubiquitin-proteasome system is actively involved in a wide range of diseases especially in cancers and neurodegenerative diseases. Understanding the working mechanism of the UPP system has driven toward development of targeted therapy in diseases involving the UPP system. Several probing systems have been developed to decipher the mechanism of ubiquitin-proteasomal systems. The probes are either designed to target ubiquitinating enzymes (E1 and E2) or 26S activity (Leestemaker and Ovaa 2017). The aberrations in the UPP system have been famously linked to neurodegenerative diseases such as Alzheimer's disease (Oddo 2008), brainstem Lewy bodies (LBs), Parkinson's disease (Olanow and McNaught 2006), ALS (Bendotti et al. 2012), nuclear inclusions, etc. (Lehman 2009). The UPP is reported to be involved in such diseases either directly or indirectly. For example, studies have indicated an active participation of neuronal UPP in the pathogenesis of Alzheimer's. The processing of amyloid plaques is known to be positively regulated by presenilin, which are

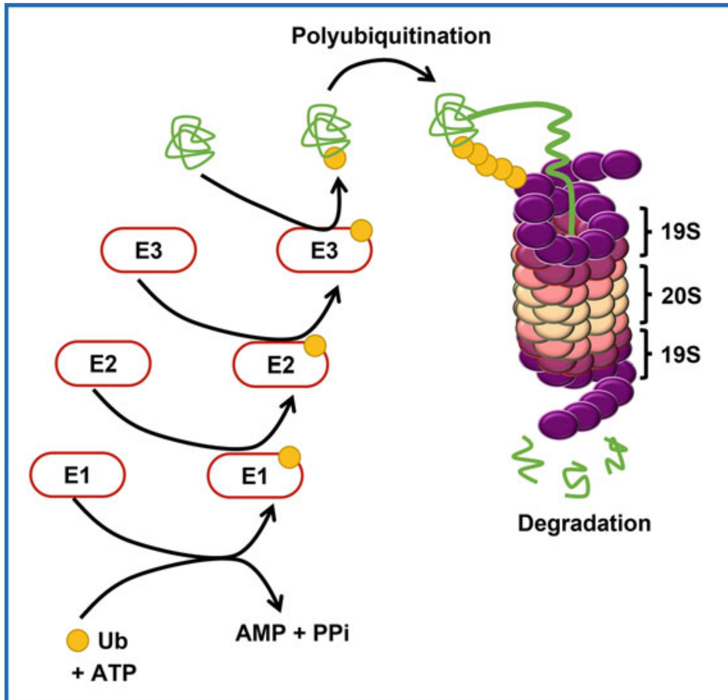

Fig. 1.7 Schematic representation of ubiquitin-proteasomal degradation pathway (UPP). The ubiquitin proteasomal pathway involves a series of ubiquitinase enzymes that partake in sequential transfer of ubiquitin (Ub) and attachment of Ub to the misfolded/foreign protein. These enzymes bind to Ub in a sequential manner through phosphorylation. Once the protein is bound to a single molecule of Ub, the Ub initiates self-polymerization and forms chain which interacts with the 19S regulatory particle of 26S proteasome. The 26S proteasome is a barrel-like structure made up of two 19S and two 20S subunits in which degradation of misfolded/foreign proteins takes place

common substrates of UPP other than ubiquitin (Gadhave et al. 2016). Similarly, the α-synuclein and Lewy bodies are commonly involved in Parkinson's disease and are reported to bind with 19S and ubiquitin implicating their respective functions (Ebrahimi-Fakhari et al. 2011; Kumar et al. 2018). The implications of key pathologies behind neurodegenerative diseases mainly lead to decrease in activity of the UPP system. This ensures the accumulation of fibrils and protein aggregates, one of the major characteristics of neurodegenerative diseases. In contrast to this, elevated response of the UPP system has been reported in many types of cancer (breast cancer, ovarian cancer, colon cancer) (Liu et al. 2015; Hyer et al. 2018).

The UPP is known to regulate cell proliferation and survival in cancerous cells. In a normal healthy cell, ubiquitination and degradation of proto-oncogenes maintain the homeostasis. However, alterations in this process lead to accumulation of proto-oncogenes during tumorigenesis and lead to uncontrolled growth of the tumor cells. For example, cancer-promoting proteins such as Nmyc, c-Myc, C-Fos are common

targets for ubiquitination and are associated with severe cancer malignancies (Paul 2008). In addition, UPP system is also involved in maintaining the normal homeostasis of the human body in several diseases. It is an essential part of the immune response system and is targeted for treatment of several diseases (Cetin et al. 2021). For example, destabilization of p53, p21, and p27, which are normally found in stable bound conditions with Mdm2 (a ubiquitin) promotes the apoptosis and inhibition of the cell cycle. Similarly, proteasome inhibition of Bcl-2 by Bas also leads to promotion of apoptosis (Peng et al. 2020). The UPP system is further actively involved in elimination of pathogens during infection (Heaton et al. 2016; Yang et al. 2019) and in regulation of both innate and adaptive immune responses. The molecular recognition of foreign particles by PAMPs that lead to activation of NF-kB and IRF3/IRF7 response is highly dependent on the UPP system (Taylor and Mossman 2013; Mitchell et al. 2016). Moreover, the UPP system is also involved in antigen presenting via class I MHC antigen. The involvement of the UPP system in various diseases and their specific roles are discussed in the following chapters.

1.11 PPIs in MODY (Maturity Onset Diabetes of the Young) Family

Maturity onset Diabetes of the young or MODY is a rare form of autosomal dominant disorder which is different from Type I and Type II diabetes (Fajans and Bell 2011). It is a noninsulin-dependent variant of diabetes which occurs in adolescents or young adults before the age of 25 years. Eleven genes have been identified till date as members of MODY family. Mutations in these genes lead to disruption in insulin production. MODY I, MODY II, MODY III, and MODY V are the common forms caused by mutation in HNF4A, GCK, HNF1A, and HNF1B respectively. MODY IV and MODY VI–XI are caused due to mutations in PDX1 (pancreatic and duodenal homeobox 1), NEUROD1 (neurogenic differentiation 1), KLF11 (Krüppel-like factor 11), CEL (carboxyl ester lipase), PAX4 (paired box gene 4), INS (insulin), and BLK (B-lymphocyte kinase) respectively. Moreover, two more genes—ABCC8 (ATP-binding cassette, subfamily C, member 8) and KCNJ11 (potassium channel, inwardly rectifying, subfamily J, member 11) are also known to cause MODY (MODY XII and XIII). All the latter mentioned proteins directly influence the production and secretion of insulin (Ozougwu et al. 2013; Urakami 2019). It is commonly misdiagnosed as Type I and Type II diabetes, and therefore a precise diagnosis is crucial for optimal treatment of the patients. GCK (glucokinase) mutations cause a mild, stable, and fasting hyperglycemia, which is asymptomatic and does not require specific treatment (Osbak et al. 2009). While mutations in HNF1A/4A (hepatocyte nuclear factor) cause pancreatic beta-cell dysfunction, hyperglycemia which results in severe vascular complications (Colclough et al. 2013; Bellanné-Chantelot et al. 2016). Further MODY V is associated with renal and genital tract abnormalities, liver dysfunction, and pancreatic agenesis.

Mutations in MODY-related genes can be missense, nonsense, frameshift, splice site, and promoter mutations. Till date, 620 GCK mutations and 414 mutations have

Table 1.7 Type and molecular function of proteins involved in different MODY variants

MODY type	Protein	Type of protein	Function of protein
MODY I	HNF4A	Transcription factor	Gene expression in hepatic and pancreatic cells
MODY II	GCK	Cytoplasmic protein	Glucose utilization by beta cell and liver
MODY III	HNF1A	Transcription factor	Regulates tissue-specific genes expression in liver and pancreatic cells
MODY IV	PDX1	Transcription factor	Glucose-regulated transcription of insulin
MODY V	HNF1B	Transcription factor	Regulates development of embryonic pancreatic cells
MODY VI	NEUROD1	Transcription factor	Regulates cell differentiation pathway in pancreatic islet cells and enteropancreatic cells
MODY VII	KLF11	Transcription factor	Enriched transcription factor in pancreas
MODY VIII	CEL	Cytoplasmic protein	Stimulates digestion of triglycerides by activation of pancreatic lipase and colipase
MODY IX	PAX4	Transcription factor	Regulation of differentiation and development of islet β-cells
MODY X	INS	Cytoplasmic protein	Glucose metabolism
MODY XI	BLK	Cytoplasmic protein	Involved in differentiation and development of B-lymphocyte and signaling
MODY XII	ABCC8	ABC transporter	Modulator of ATP-sensitive K^+ channels and insulin release
MODY XIII	KCNJ11	Receptor	Regulates the expression of ATP-sensitive K^+ channel

been reported in case of MODY I and MODY III (Anik et al. 2015). The physiological functions of all MODY-related genes are mentioned in the Table 1.7. As mentioned, MODY-related proteins are key enzymes and transcription factors involved in development of pancreas, liver, genital tract, and renal architecture. Mutations in any of the proteins alter the PPI network and are crucial for MODY pathogenesis. Several works have been conducted to understand the stereochemical and functional aspects of the MODY protein using experimental and computational analysis (Sneha et al. 2018; George et al. 2014; Sneha and Doss 2017). Sneha et al., performed a computational analysis aimed to decipher the interacting pathway of the MODY-causing protein using the PPI analysis network. Authors reported that all 11 proteins were interacting partners of essential transcription factors, and these proteins are involved in the development of the organs as well as regulation of glucose in the body. HNF4A was reported to interact with GLUT2, catenin beta-1 (CTNNB1), apolipoprotein A-I (APOA1), HNF1A, GCK, FOXA2, and other essential proteins. FOXA2 is a transcription factor involved in expression of genes vital for glucose sensing in pancreatic beta cells. Similarly, GCK was found to be

interacting with ten key enzymes involved in glucose sensing and insulin secretion during glucose homeostasis. Some of these proteins are phosphoglucomutase (PGM1 and PGM2), glucose-6-phosphate isomerase (GPI), trehalase, FOXA2, and glucose-6-phosphatase (Sneha et al. 2018). Similar results have been reported by Nihitha et al. through a computational analysis study (Nihitha et al. 2018). The understanding of PPIs involved in MODY are useful in development of treatment options (Delvecchio et al. 2020). For example, in a recent clinical study, GLP-1 receptor agonist therapy has been proven effective in case of HNF4A MODY (Broome et al. 2020). In general, sulfonylureas are the classic treatment options in all commonly reported MODYs (Urbanova et al. 2015).

1.12 Conclusion

Protein–protein interactions are the mediators of the smooth functioning of cellular machinery. Genetic mutations, epigenetic changes, conformational interfaces, and multiple binding partners are the key modulators of protein–protein interactions in humans. In a normal healthy individual, the protein–protein interactions regulate the homeostasis. Dysregulation of such interactions is highly responsible for defects in molecular pathways and a diseased state. This chapter briefly discusses various elementary proteins and their interactions in a cell and how it affects the fundamental cellular response. The understanding of correct protein interaction networks and molecular pathways paves an enlightened path for investigation of differential changes in key PPI involved in a variety of diseases such as cataract, cystic fibrosis, MODY, and GPCR-related diseases.

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Protein–Protein Interactions in Cancer

2

2.1 Introduction to Cancer

Cancer is a condition where abnormal cellular growth takes place in tissues. In contrast to normal cells, the cell signaling circuit in cancer cells goes awry resulting in uncontrolled proliferation (Weinberg 1996). The unfettered proliferation disrupts the homeostasis leading to weakening of the immune system and a diseased state. Upon proliferation, cancerous cells accumulate, and form cell mass called malignant tumors. Another property of tumor cell is their ability to metastasize from their origin and migrate to other organs. Cancer is classified into five types based upon the affected organs: carcinoma, sarcoma, melanoma, lymphoma, and leukemia. A variety of physical and biological agents act as carcinogens, i.e., cancer-causing agents. Genetic anomalies, epigenetics, and aging also play an influential role in the initiation and spread of cancer in the human body. Genetic mutations perturb the functionality of proteins thereby changing the cellular and molecular activity. Several sets of genes called proto-oncogenes (triggers tumor), and tumor-suppressing genes are predominantly involved in proliferation of cancer (DePinho 2000).

These sets of genes are normally involved in the cell cycle checkpoints and regulate a variety of cellular functions. The occurrence of carcinogenesis is led by mutations such as point, frameshift, nonsense mutations, and chemical damage to the DNA. The chemical aberrations and mutations lead to chromosomal rearrangements, epigenetic modifications, overexpression, or low expression of a particular gene. These modifications result in formation of chimeric, truncated, or misfolded protein products which dysregulate cellular functions. The aberrant genetic change divides genes into two basic classes: (1) mutations which increase the carcinogenic activity of encoded protein in case of proto-oncogenes, or (2) mutations that lead to inactivation of gene function as in the case of tumor-suppressing genes (Bertram 2000). Mutations need to happen in an accumulative manner for progression of carcinogenesis. The tumor development occurs in five stages as shown in Fig. 2.1. Tumor

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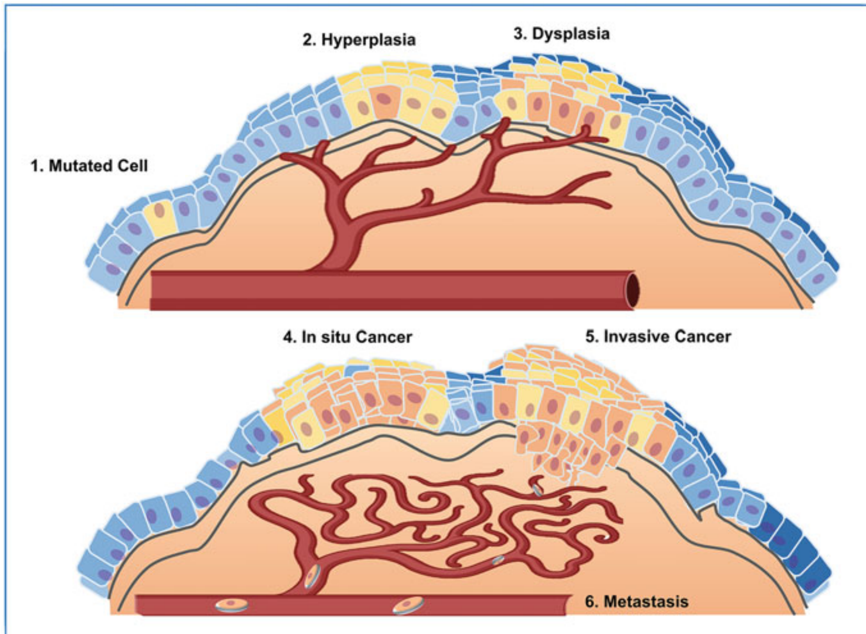

Fig. 2.1 Stages of cancer progression. The cancer progression starts when a single cell gets mutated and starts proliferating (hyperplasia) uncontrollably. Further the metabolic secretions from mutated cells start affecting the nearby cells and cell morphogenesis is initiated (dysplasia). Dysplasia all together with an elevated uncontrollable growth result in formation of in situ cancer, which further turns into an invasive cancer and metastasizes into the blood vessels for the formation of tumor in other organs of human body

development starts when a genetically altered cell and its descendants reproduce at a higher rate than normal, thus creating a condition called hyperplasia. Further mutations take place, followed by elevation of cell proliferation and change in cell shapes, in the stage known as dysplasia. The growth and shape of the abnormal cells become more atypical and develop into in situ cancer. The in situ cancer turns into invasive cancer, once it damages tissue barrier and metastasizes to other organs through blood vessels (Torpy et al. 2010).

The tumor shifts the nearby paradigm to accommodate the survival of tumor. Tumor microenvironment plays a great role in deciding the fate of altered cells. The tumor microenvironment (TME) is a densely packed space in which the tumor cells reside and proliferate till it metastasizes. Dense population makes the TME hypoxic, as the tumor outgrows the diffusion limit of blood supply. This results in an altered metabolism and usage of secondary substrates for biosynthetic pathways. This phenomenon is known as the Warburg effect. The Warburg effect initiates angiogenesis, oncogene activation, altered biochemical pathways. The altered pathways in the TME are greatly responsible for generation of toxic byproducts and act as a

positive feedback loop for themselves (Hsu and Sabatini 2008; Hanahan and Weinberg 2011).

Molecular interactions such as protein–protein, protein–DNA interactions are important factors in deciding the fate of cancer cells. Over the years, increasing number of point mutations, and protein–protein interactions have been deciphered. These interactions are largely responsible for dysregulation of biochemical pathways. Cancer poses many challenges in the form of multiple mutations, cellular heterogeneity, ability of epithelial-to-mesenchymal transitions (EMT), drug resistance, and individual specific symptoms. All these properties of cancer are regulated through a variety of signaling pathways and protein–protein interactions. Hence, aberrant interactions and their role in cancer prognosis are important for nitpicking of the faulty pathways and understanding the cancer progression. Moreover, it boosts the recognition of novel biomarkers and development of targeted anticancer therapy. This chapter gives a brief overview of major proteins, PPIs, and their inhibitors involved in the diagnosis and treatment of cancer.

2.2 Profiling PPIs of Single Cancer Cells

The cellular heterogeneity is one of the unique features of tumor cells (Krebs et al. 2014). Every cell carries a definite genome and is programmed in an exclusive manner. Moreover, depending on the spatiotemporal placement of the cancer cell, the gene expression of each cell is markedly variable (Swanton 2012). The striking variability in the tumor environment offered the innovation of a remarkable method for identification of biomarkers and spatiotemporal changes in cancer patients. This technique is known as single-cell profiling (SCP) which embodies the gene, protein, and metabolite expression analysis of a single cell (Krebs et al. 2014). Profiling of single cancer cells gained traction since 2013, and was acknowledged as the method of the year (Eberwine et al. 2014). The profiling involves recognition of novel mutations, structural changes, and metabolite expression. The data gained through SCP can be used to trace the evolution and clonal structure of the cell. The severity of mosaicism in somatic cells and their functional consequences can also be studied with the help of single-cell profiling. Most of the research in the field of cancer biology has been centered on the identification of population-based biomarkers, and broad-spectrum anticancer drugs. However, the arbitrary stimulation of genetic and epigenetic changes and reaction to anticancer molecules differ from patient to patient as well as in various cancers. The single-cell profiling dissects this heterogeneity. Moreover, this technique offers an unbiased way to detect relevant differences in cells at the genetic and proteomic level even when they cannot be differentiated by known biomarkers or morphologically. Besides cancer diagnostics, SCP makes sequencing of rare cells more accessible where spatiotemporal properties play a decisive role.

Molecular profiling of circulating tumor cells (CTCs) is one of the well-documented methods where SCP has been proven as a powerful tool for cancer diagnostic and personalized cancer therapy. The CTC trafficking suggests that the

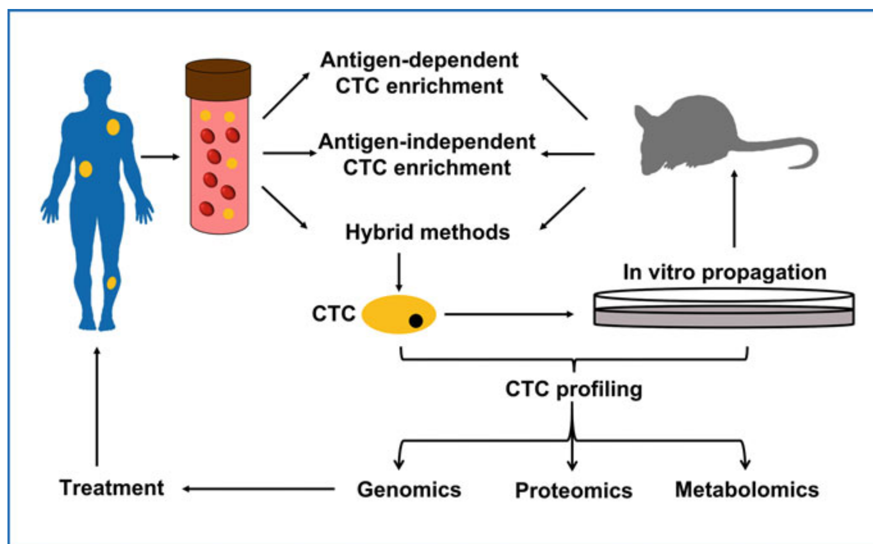

Fig. 2.2 Schematic representation of single-cell profiling and its use for cancer diagnostic and treatment. Blood sample containing circulating tumor cells (CTC) is collected from the patients. The CTCs are isolated through three different methods (Antigen-dependent, antigen-independent, hybrid methods, and in vitro/in vivo propagation). Once the CTCs are isolated, the genome, proteome, and metabolome of CTCs are profiled via various biochemical and biophysical assays for development of targeted therapy and diagnostics

tumor cells migrate between primary tumor, bone marrow, and metastases in advanced stages of cancer. CTCs have the potential to develop from multiple sites of a tumor, thereby representing several sub-populations of **CTCs** (Kim et al. 2009). Several cases of breast and lung cancer (Kapeleris et al. 2018; Cristofanilli et al. 2019; Yousefi et al. 2020) have been reported to exhibit subpopulations of CTCs. The profiling of CTCs has been proven to be useful in CTC characterization, prognostic, and pharmacodynamic biomarkers (Boral et al. 2020; Kalita and Coumar 2021; Nel et al. 2021). Above all, the profiling of tumor cell heterogeneity increases the efficacy of targeted therapy to a great extent, as it is dictated by identification of specific biomarkers. This reduces the chances of drug resistance, their concomitant effects, and cancer relapse. For instance, tumor heterogeneity plays a significant role in resistance toward targeted therapy in BRAFV600E melanoma patients, where secondary resistance is well-known (Marusyk et al. 2012; Das Thakur et al. 2013). CTCs profiling is a multifaceted process involved in precise cancer diagnostics. The whole process is initiated with CTCs' enrichment techniques for cloning of CTCs populations leading to genetic, protein, and functional analysis (Labib and Kelley 2021). This information gained is then utilized to design personalized therapies for cancer patients (Fig. 2.2). Various methodologies have been developed for profiling of CTCs such as cell enrichment methods (Song et al. 2017), CTC cluster assay, and MagRC approach (Labib and Kelley 2021).

The SCP analysis for cancer is categorized into four parts based upon the components of the cell studied. These are cell-based, nucleic-acid based, protein-based, and lipid and metabolite-based analysis (Stuart and Satija 2019). The cell-based analysis is popularly performed with the help of flow cytometry, mass cytometry, and live cell tracking. Flow cytometry and mass cytometry are powerful multidimensional techniques that mediate the monitoring of cellular events or a whole cell. However, both techniques lack in providing the spatiotemporal information. The live cell tracking alleviates this disadvantage and offers a dynamic procedure for detection of cancer cells based upon their interaction with dyes and fluorescent proteins. Live cell tracking is also used to observe the anticancer drug response against tumor cells. While cell-based analysis allows probing of membrane interactions and viability of a single cell, the variation in the gene expression and protein function is estimated by nucleic acid and protein-based methods. Nucleic acid and protein-based analysis are arduous processes due to small input material. However, PCR techniques such as SINCE-PCR, and microfluidic systems such as C1 single-cell auto prep system have proven as high-throughput sensitive techniques for estimation of changes in DNA and mRNA levels in cancer cells. Meanwhile techniques like microfluidic devices, microfluidic image cytometry (Prakadan et al. 2017), MagRC, mass cytometry platform (CyTOF) (Chen et al. 2019) have been reported as high-precision techniques for tumor cell protein analysis. CyTOF has been reported to feature the phenotypic heterogeneity in acute lymphoblastic leukemia (ALL) (Khoo et al. 2016). Recently, a method of single-cell protein profiling has been reported, where a microfluidic method with barcoded beads was developed that expedites capture, data acquisition, and quantification of proteins from individual breast cancer cells (Armbrecht et al. 2019). Similarly, Ryu et al., have profiled protein–protein interactions in lung adenocarcinoma cells using in situ lysis and immunoprecipitation. The study revealed high heterogeneity in EGFR signaling and different patterns of PPIs in PC9 lung adenocarcinoma cells (Ryu et al. 2019). The heterogeneity of PPIs in stage III/IV nonsmall cell lung cancer (NSCLC) has also been reported by assessment of single-cell resolution profiles and protein interaction network analysis. The study also revealed the correlation of tumor incongruity with tumor-associated neutrophils and NK cells (Wu et al. 2021).

2.3 Building Cancer Cell Maps

Cells are intricate machines made up of genetic blueprints formed by mutation and evolution, whose information is expressed in response to the cellular environment. Despite the fact that every human cell has the same DNA, sophisticated regulatory networks govern the expressed genes, resulting in the wide range of specific cell types which make up our body. Hence, we need to investigate gene expression and cell structure apropos of the events that drive cell behaviors to acquire a better understanding of how cells work. This will give a foundation for untangling and modeling the myriad of dynamic, interacting components that make life possible. Recent initiatives, such as, the one described by Thul et al. (2017), and the new

methodologies suggest combining of genomic, epigenetic, and structural studies to create a whole cell atlas that represents the full range of cell types and states in the human body, which might help in better understanding of cell physiology (Horwitz and Johnson 2017). Building cancer cell maps has become an important technique for assessment and prediction of protein–-protein interactions and intracellular protein–drug interactions. The system biology approaches (Kuenzi and Ideker 2020), machine learning, and artificial intelligence algorithms (Li et al. 2021) are being rigorously used for development of cancer cell maps. Recently, spatial maps were built by spatial transcriptomics of prostate cancer by Berglund et al. which revealed distinct expression profiles for different tissue regions, and provided insights into differential gene expression during progression of prostate cancer (Berglund et al. 2018). Similarly single-cell profiling is used for mapping of cancer-specific surface antigens and other proteins for construction of antigen/protein maps and further development of cancer therapeutics (Lareau et al. 2021).

2.4 OncoPPI Portal

With a jargon of cancer genomics data reported, and the emerging need of understanding how interactions occur among genes, it is necessary to create a platform to explore oncogenic PPI networks. Several compelling databases and resources have been built in the last few decades for extensive characterization and annotation of cancer-related genes based on genetic changes in cancer patients and individual cell lines (Cerami et al. 2012; Gao et al. 2013; Collisson et al. 2014). Furthermore, gene functional interconnection in cancer cells has been predicted using extensive bioinformatics assessments of mRNA expression and cell line sensitivity for individual gene knockouts. Identifying and prioritizing oncogenic PPIs for comprehensive functional research still remained as a bottleneck. To solve this, a high-throughput screening platform called OncoPPi portal has been created. OncoPPi Portal is an integrative resource platform, and database used for high-throughput screening of PPIs between cancer-associated proteins (Ivanov et al. 2018; Ivanov 2020) (Fig. 2.3).

The platform is an interactive web resource that grants investigators access, manipulate, and interpret highly specific cancer protein interaction networks for biological studies. The platform provides network connectivity analysis, mutual exclusivity analysis of genomic mutations, colocalization of interacting cellular partners, and domain–domain interactions information of PPIs. It further allows the user to inspect the functional impact of PPIs on cancer cell physiology and enables the discovery of novel tumor-related networks, druggable targets (Ivanov 2020). More than 260 cancer-associated PPIs have been identified with this portal and reveal new regulatory mechanisms for cancer genes such as MYC, STK11, RASSF1, and CDK4. Similar to TGCA, OncoPPi portal facilitates the identification of pan cancer regulatory networks by analyzing differential protein–protein interactions. Recently, several important breast cancers related PPIs have been identified using the OncoPPi portal and various other cancer databases. The study revealed 140 essential genes such as RAC1, AKT1, CCND1, PIK3CA, ERBB2,

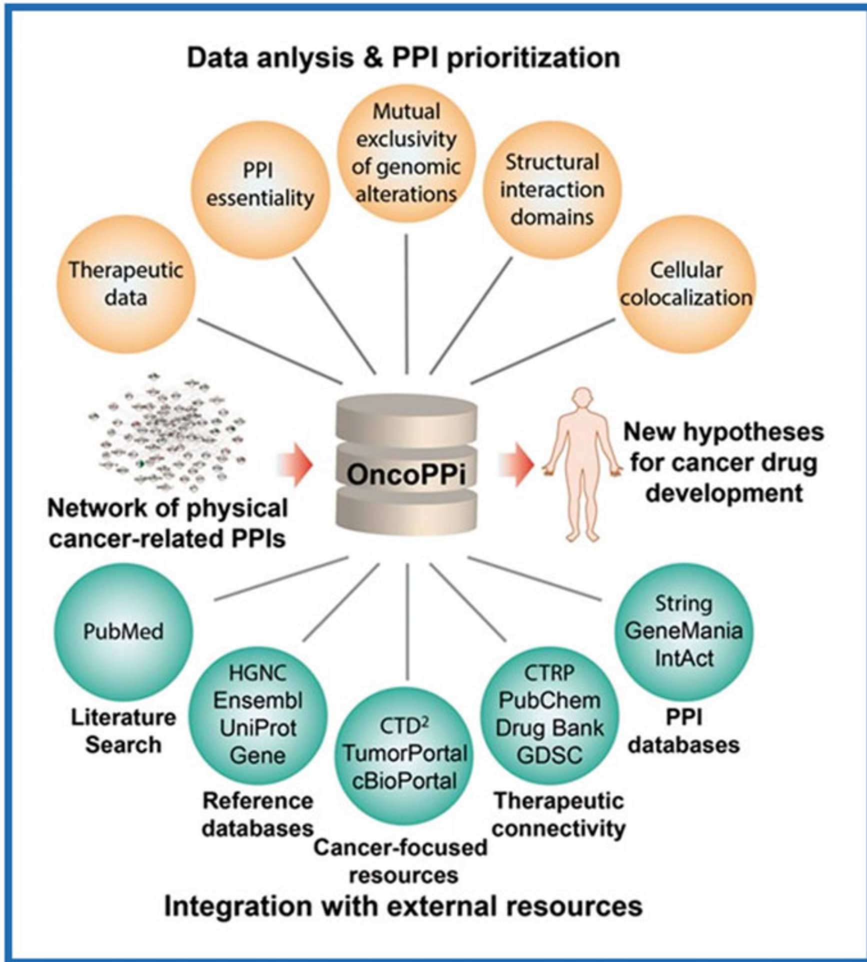

Fig. 2.3 Schematic overview of various resources provided by the OncoPPi portal for assessment of protein–protein interactions involved in different types of cancers. (Adapted from Ivanov et al. 2018)

CDH1, MAPK14, TP53, MAPK1, SRC, RAC3, BCL2, CTNNB1, EGFR, CDK2, GRB2, MED1, and GATA3. It further revealed that the most altered signaling pathways associated with breast cancer were Her-2-enriched and basal–like-associated signaling pathways (López-Cortés et al. 2020).

2.5 PPIs Between Growth Factors/Chemokines and Their Receptors

Tumors are convoluted microenvironment (TME) composed of a variety of cell types that coexist and interact with one another through a complex signaling network. This involves chemokine and cytokine cross talk between tumor cells, as well as the effects of these chemokines and cytokines on the immune response and metastasis. Chemokines are chemotactic proteins associated with immune cell trafficking and inflammatory responses in malignant diseases. The spatiotemporal expression of chemokines controls the directed migration of cells. Chemokines are simple proteins that bind to glycosaminoglycans, which are essential in their biology. The chemokines are separated into four subfamilies—CC, CXC, CX3C, and XC based on the position of the first two amino-terminal Cys residues (Poluri 2014). Nearly 50 chemokines, 20 signaling chemokine receptors, and four AKCRs have all been discovered thus far. Differential chemokine receptor expression on leukocytes leads to the selective recruitment of certain cell types under specific situations, resulting in suitable and effective immune responses customized to the infecting pathogen or external stimulus (Tripathi and Poluri 2020). Chemokines regulate cellular motility and intercellular interactions, and so have a significant impact on tumor formation. Different chemokines are released by neighboring tumor cells and cancer cells in the TME, resulting in the accumulation and stimulation of antitumor and protumor responsive cell types (Gulati and Poluri 2016). Chemokines have a number of roles in the proliferation and survival of cancer cells. When chemokine receptors on cancer cells are ligated, the MAPK/Erk signaling cascade is initiated, culminating in the activation of key growth-stimulating genes like cyclin D1, Fos, and heparin-associated EGF. Chemokines can also help cancer cells survive by altering the balance of proapoptotic and antiapoptotic proteins in the cells, such as upregulating Mdm2 and downregulating Bcl-2 expression of suppressing caspase-3 and caspase-9 activation (Mollica Poeta et al. 2019). Tumor cells are also capable of producing and expressing growth-promoting chemokines and receptors. Melanoma, for example, has been found to express several chemokines connected to tumor progression and growth, including GRO chemokines (CXCL1/2/3), IL8 (CXCL8), MCP-1 (CCL2), and CCL5. Tumor cells also upregulate chemokine receptors which result in a feedback loop where cancer cells divide faster in response to TME-available growth-promoting chemokines. CXCR4, for example, is expressed frequently on breast cancer cells but not on breast epithelial cells. Further, upregulation of CXCR4 expression induces cancer cells to respond to CXCL12, its cognate ligand (Chow and Luster 2014; Kawaguchi et al. 2019).

Beside tumor growth and progression, chemokines are actively involved in angiogenesis, metastasis, cancer immunology, and cancer therapy. Chemokines are transported all over the body through the lymphatic system. Hence, they portray an essential part in angiogenesis. The survival of tumor cells greatly relies on the presence of adequate oxygen and nutrients. Hence cells within the tumors are tremendously dependent on the adjoining blood vessels making angiogenesis a key rate-defining step in tumor formation and progression. Various chemokines

and its receptors are associated with the regulation of tumor angiogenesis. ELR+ chemokines such as CXCL1, CXCL2, CXCL3, and CXCL8, their receptors CXCR1 and CXCR2 are potent angiogenic chemokines (Jaffer and Ma 2016). While ELR-chemokines (CXCL4, CXCL9, CXCL10) have been shown to be inhibitors to angiogenesis with an expedition of CXCL12, which is known as the most potent angiogenic factor (Tokunaga et al. 2018). Proangiogenic chemokines increase the migration and proliferation of endothelial cells by binding to chemokine receptors present on the surface of endothelial cells. CXC chemokines such as CXCL8 and CXCL12 upregulate the expression of VEGF, which enhances the production of proangiogenic chemokines. In contrast angiostatic chemokines such as CXCL4 and CXCL10 are involved in suppression of VEGF-induced and FGF-induced angiogenesis. Further, they are involved in the trafficking of angiostatic CXCR3-expressing CD4+ T-helper (Th1) cell and CD8+ cytotoxic T cells (Cannon et al. 2021). Similarly, CXCR4 and its ligand CXCL12 have been linked to cancer spread. The CXCR4/CXCL12 axis is blocked to prevent breast cancer from spreading to the lungs. It has also been related to cancer metastasis, including prostate cancer, lung cancer, and glioblastoma. CXCR4 expression has been linked to increased cancer metastasis in humans (Wang et al. 2016). CCR7 and its ligand CCL21 are required for tumor cell entry into lymphatic arteries in dendritic cells (DCs) and T cells, and CCR7 and its ligand CCL21 may also play a function in cancer cells (Rizeq and Malki 2020). CCL1 has also been shown to attract CCR8-positive tumors when generated by lymphatic endothelial cells in subcapsular sinus (Korbecki et al. 2020).

Immune cells play an influential role in tumor development. Since the primary goal of chemokines is leukocyte trafficking, it is tenaciously involved in tumor-residing immune cell composition by recruitment of leukocytes in TME (Balkwill 2004; Mantovani et al. 2010). The immune cells can either be effector cells, i.e., involved in removal of cancer cells or promoter cells. Such immune cells are popularly utilized as positive prognostic indicators of various stages of cancer. The Th1 immune response is strongly connected to CXCR3, as well as its ligands CXCL9 and CXCL10. The sensitivity of the Th1-biased immune response determines the efficiency of an antitumor immune cell response. Recent studies have indicated that CXCR3-mediated anticancer responses are achieved by recruitment of NK cells, CD4+ Th1 cells, and CD8+ cytotoxic T lymphocytes (CTL) into tumors (Kim et al. 2018). Because type I and II IFNs enhance CXCL9 and CXCL10 expression, recruited lymphocytes-derived IFNs can amplify intratumor CXCL9 and CXCL10 expression, inhibiting tumor growth. Recently, CXCR3 has also been discovered to play an important function in macrophage polarization. In a mouse breast cancer model, CXCR3 deletion causes macrophages to polarize toward an M2 phenotype, which promotes tumor growth; this result showed that CXCR3 is necessary for M1 macrophage formation. Depending on the activating stimulus from local cytokine milieu, macrophages are categorized into M1 and M2. Macrophage M1 are stimulated by IFN whereas, M2 are trafficked more effectively by IL4, IL13, and TGF (Zhu et al. 2015). M1 macrophages are tumoricidal and can operate as antigen-presenting cells to activate effector T cells. M2 macrophages, on the other hand, boost Th2 cell responses and produce a lot of IL10 and protumorigenic

chemicals, which help tumors grow and spread. CCR5 and its associated chemokine, CCL5 play critical roles in the recruitment of antitumor leukocytes. CCR5 deficiency elevates development of pancreatic adenocarcinoma, transplantable Lewis's lung adenocarcinoma, and lymphoma in EG7 mice. CCR5 expression on CD4+ and CD8+ T cells is required for tumor-protective immunity to develop. CCR5-expressing CD4+ T cells can promote APC maturation via the CD40/CD40L pathway, resulting in a maximal antitumor response from CD8+ T cells (Korbecki et al. 2020).

Owing to the relevance of chemokines in tumor physiology, therapeutics targeting chemokines are extensively studied (Mollica Poeta et al. 2019). Targeting the immune system is a practical strategy to cancer treatment moreover, several ways have been devised to improve leukocyte antitumor activity. Owing to this, chemokines and chemokine receptors are implicated in numerous areas of cancer biology, their potential targeting has been investigated in numerous preclinical investigations and clinical trials. For hematological malignancies, an anti-CCR4 monoclonal antibody (Mogamulizumab) and a CXCR4 antagonist known as AMD3100 are under clinical use. Several CC chemokines and their receptors such as CCL2, CCL3, CCR1, CCR2, CCR4, and CXC chemokines such as CXCL12 and its receptor CXCR4 have been extensively studied for development of antitumor agents. In a mouse model of breast cancer, the CCR1 receptor antagonist CCX9588 was coupled with anti-PD-L1, indicating a synergistic antitumor effect through decreasing myeloid infiltration (Karin 2018). Because CCR1 antagonists demonstrated no adverse effects in individuals with autoimmune illness, they are promising candidates for regulating the myeloid infiltration in combination therapy. Similarly, tweaking the CCL2-CCR2 axis also exhibits the antitumoral efficacy in various malignancies by reduction of infiltrating protumorigenic and prometastatic monocytes. Several CCR2 inhibitors such as PF-04136309 have been identified for oral treatment of pancreatic tumors. When used alone in a preclinical model, the inhibitor PF-04136309 reduced the quantity of TAMs and had a minor effect on tumor evolution, but it functioned synergistically with the chemotherapy medication Gemcitabine. Recent results from a Phase Ib/II trial with pancreatic cancer patients in which PF-04136309 was administered together with nab-Paclitaxel, a nanoparticle albumin-bound version of nab-Paclitaxel or PTX behaves as a stimulus for TAM activation toward an M1-like phenotype, and gemcitabine (Xu et al. 2021). Another CCR2 inhibitor, CCX872, has shown great potential in the treatment of pancreatic cancers. It boosted the efficacy of anti-PD-1 chemotherapy in a preclinical model, and positive findings were obtained in a clinical trial, when administered in combination with FX (Linehan et al. 2018). Several inhibitors and their mechanisms are elaborately discussed in Chap. 7 of this book.

2.6 PPIs for Cytoskeleton Dynamic Pathways

Cytoskeleton performs a multitude of functions to maintain cellular homeostasis. It is a mesh-like dynamic network of protein filaments present in the cytoplasm (Hohmann and Dehghani 2019). The primary role of cytoskeleton is to provide structural stability to the cell. Moreover, the cytoskeleton is involved in signal transduction, cell migration, cellular division (segregation of chromosomes and cytokinesis), cell wall formation, endocytosis, and intracellular transport of biomolecules. In eukaryotes, the cytoskeleton is composed of microfilaments, intermediate filaments, and microtubules. Microfilaments and intermediate filaments are polymers of actin (~7 nm in width), which are the ubiquitous protein in eukaryotes. While microtubules are made up of tubulin protein. Both actin and tubulins are extremely adaptable, dynamic polymers (Dominguez and Holmes 2011). They provide skeletal support to cytoplasmic organelles and intracellular compartments, define cell polarity, and generate movement-related motion (pushing and contractile forces). Both the cytoskeletal structures facilitate chromosomal separation, and cell division during the cell cycle. They affect cell shape and polarity during morphogenesis (Spichal and Fabre 2017). Moreover, actins and tubulins encourage steady cell-cell and cell matrix adhesions via interactions with cadherins and integrins respectively. Finally, during cell migration, protrusive forces at the front and retraction forces at the back are mediated by the cytoskeleton as well. All of these are the essential elements of the cell behavior that frequently go wrong in cancer, thus emphasizing the relevance of cytoskeleton dynamics in cancer cell biology (Olson and Sahai 2009; Schiewek et al. 2018; Hall 2009).

The role of cytoskeleton in cancer cell biology can broadly be categorized by three cytoskeletal functions. These are cell cycle, morphogenesis, and migration (Bendris et al. 2015). The modulation of cytoskeletal architectures is an essential process at each stage of the cell cycle process. The promotion of unrestrained growth depends on the various cell cycle-related proteins. Proteins such as CDKs directly or indirectly interact with actin filaments at G2, S, and M phases of cell cycle for cell cycle progression. One of the fundamental steps—chromosomal segregation is highly dependent on the cytoskeletal dynamics (Po'uha and Kavallaris 2015). Moreover, the cytoskeleton also mediates the cell-cell, and cell-matrix adhesion that modulates the cell cycle machinery through specific checkpoints. The cyclin-CDK complexes and the phosphorylated cytoskeleton proteins regulate the assembly and formation of the actin network (Bendris et al. 2015). These interactions not only mediate cell proliferation, but also facilitate cell morphogenesis. Cell morphogenesis is an important part of the cancer metastasis process. The transition of epithelial cells into mesenchymal cells is an important factor in cancer metastasis (Ribatti 2017). For metastasis, it is important that the cancer cell changes its molecular and structural architecture such that it can survive the transportation process from the site of origin to other organs. Being the fundamental member of the structural architecture of a cell, the cytoskeleton is a prerequisite feature of morphology, migration, and invasion of cancer cells. Beside actin, the microtubule network is the driving force in case of cell migration. Moreover, intermediate filaments are significantly rearranged, and

their molecular architecture shifts from cytokeratin-rich to vimentin-rich networks during endothelial-mesenchymal transition (Pastushenko and Blanpain 2019). Further, actin-binding protein-regulated spatiotemporal changes in actin filament's polymeric state and formation of lamellipodia, filopodia, and pseudopodia are used by cancer cells during invasion and metastasis process (Aseervatham 2020).

2.7 Ras–Raf Interactions

The Ras-Raf-MEK (MAPK-ERK kinase)-ERK (extracellular signal-regulated kinase) pathway is a fundamental signaling pathway in all eukaryotes. It governs differentiation, proliferation, survival, aging, and death of cells. The signaling pathway involves Ras as G-protein and Raf as MAPKKKs (mitogen-activated protein kinase kinase kinase), MEK as MAPKK (MAPK kinase), and ERK as MAPK. The activation of all kinases takes place due to phosphorylation of Ser/Thr residues-rich domain. Upon activation, the MAPKs regulate the cellular functions by activation of transcription factors. Both Ras and Raf genes are identified as proto-oncogenes, thereby essential for cell proliferation, growth, and progression of cancer. Further, the ERK pathway is a nonlinear pathway, i.e., a variety of proteins interact with protein of ERK pathway. Such a cross talk is important for normal functioning of the Ras-Raf pathway and maintains the same using positive and negative feedback mechanisms (Fig. 2.4). The prevalence of Ras–Raf interactions in cancer has been extensively studied over the years. This section discusses the role of Ras–Raf interaction in normal and cancerous cells (Zebisch et al. 2007).

2.7.1 Ras Protein

Ras is a 21 kDa plasma membrane GTPase that transmits signal in GTP-bound state. Ras proteins are binary switches similar to Rho-GTPases, and alternates between ON and OFF state during signal transduction (Wittinghofer and Pal 1991). The Ras protein is found in three isoforms—N-, Ha-, and Ki-Ras and is highly conserved in eukaryotes (Castellano and Santos 2011). The interaction of extracellular ligands with GPCRs initiates the alteration of inactive GDP-bound forms to active GTP-bound state with the help of guanine nucleotide exchange factors (GEFs—SOS, CDC25, SDC-25). The transformation from active to inactive state is catalyzed by GTPase-activating protein (GAPs: IRA-1, IRA-2). The GEF-GAP dependence allows regulation of Ras activity and the downstream signaling. Moreover, regulation of Ras activity also depends on its inherent GTPase activity, and translocation to the plasma membrane. Ras is activated by GEFs such as SOS (son of sevenless). Ras in its activated form is translocated to the plasma membrane, where it targets and activates Ser/Thr-rich MAPKs known as Raf kinases. The structural studies elucidated two regions are present in Ras protein, and known as switch-I and switch-II. Both these regions are critical for its role in protein–protein interactions (Shima et al. 2010). The switch regions undergo conformational changes in

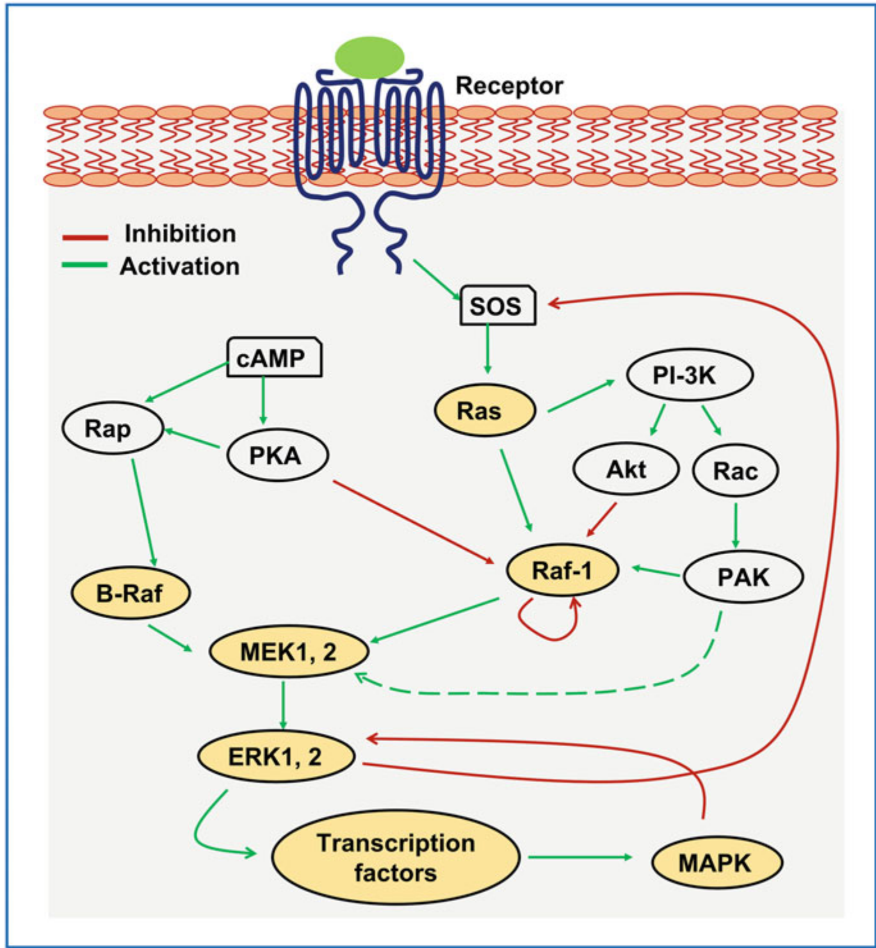

Fig. 2.4 Schematic representation of positive (green) and negative (red) feedback loops involved in regulation of Ras–Raf pathway in a cell

GTP-bound state forming a hydrogen bond between γ-phosphate of GTP, T35, and G60 residues of Ras. Intrinsically, Ras proteins remain in their inactive state due to a slow off-rate for GDP. GEFs accelerate the GDP to GTP conversion by interacting with the P loop (res 10–17) and reducing the affinity of Ras toward GDP. The GTP then binds to Ras and GEF dissociates (Simanshu et al. 2017). The GAP mediates hydrolysis of GTP in a similar fashion via binding to the GAP-related domain, GRD (res 718–1037). GAP interacts with the switch II region inducing two conformational changes. Firstly, it stabilizes Q61 by coordination with water molecule, and secondly it mediates protruding of the arginine finger (R789) which interacts with α- and β-phosphate of GDP. Ras proteins also consist of a CaaX motif in the N-terminal

region that plays an important role in insertion of Ras in cellular membranes with the help of post-translational modifications (Simanshu et al. 2017; Li et al. 2020).

2.7.2 Raf Kinases and Other Ras Effectors

Raf kinases are encoded by Raf genes and are generally found in higher eukaryotes. In mammals, three kinds of Raf proteins have been identified: Raf-1, A-Raf, and B-Raf. All three isoforms of Raf are activated by GTP-bound Ras protein, and act as upstream effectors of ERK signaling pathway. Raf-1 and B-Raf have been extensively studied in signaling pathways. The Raf proteins are multimeric proteins (300–500 kDa), with a common structure made up of three conserved regions: CR1, CR2, and CR3 (Brummer and McInnes 2020; Rezaei Adariani et al. 2018). The CR1 contains a Ras-binding (RBD) and cysteine-rich domain (CRD) for interaction with Ras and the plasma membrane. The CRD is also responsible for autoinhibition of Raf by interacting with the kinase domain. CR2 contains inhibitory phosphorylation targets which play an important role in negative regulation of Ras–Raf interaction. CR3 is the kinase domain which upon phosphorylation proceeds with the downstream signaling of ERK signaling cascade. CR1 and CR2 together constitute the regulatory domain, while CR3 features the catalytic domain of Raf proteins (Matallanas et al. 2011). The regulatory domain is an essential factor in the activation of oncogenic activity. However, in some cases, oncogenic mutations in the kinase domain of Raf have been observed to activate ERK pathway and cancer progression (Durrant and Morrison 2018; Roskoski Jr 2018). One such instance is the mutation V600E in the B-Raf kinase domain, which elevates the kinase activity by mimicking phosphorylation of CR2 sites and inhibits the negative regulation of B-Raf activity. Other than Raf, the Ras GTPases can also interact with other cellular effectors. These effectors are well studied and have been identified to be involved in cancer cells. These effectors include, PI3K, RalGDS, novel RAS effector 1A (NORE1A), Af6, Grb14 (growth factor receptor 14), PLC (phospholipase C), TIAM (T cell lymphoma invasion and metastasis-inducing protein), and RIN1 (Ras and Rab interactor 1) (Santini et al. 2019). The translocation of Ras–Raf complex to the cell membrane activates the Ser/Thr kinase activity of Raf. Upon activation, Raf acts as MAPK kinase kinase (MAPKKK), which activates MAPK kinases (MAPKK)—MEK1 and MEK2. The MEKs in turn stimulate the activation of ERK1 and ERK2 kinases by phosphorylation. Upon phosphorylation, the ERKs translocate to the nucleus, where it phosphorylates transcriptional factors responsible for cell proliferation, survival, morphogenesis, motility, and differentiation (Mysore et al. 2021).

2.7.3 Prevalence of Ras and Raf Protein in Cancer

Ras wild type and mutated isoforms are prevalent in human cancer and have been identified as a common cause for development of tumors. Most of the cancer-causing

mutations have been recorded in K-Ras preceded by N-Ras and H-Ras (Degirmenci et al. 2020; Zhou et al. 2016). Ras mutations generally occur at residues 12, 13, and 61. Glycine at position 12 or 13 when replaced by any other amino acid except proline prevents the arginine finger from protruding out and GTP hydrolysis. Similarly, mutation at Q61 position also affects the GAP-mediated hydrolysis of GTPase (Hobbs et al. 2016). Several other mutations such as A146, R164Q, and K176Q also reduce the affinity of GDP resulting in accumulation of Ras in its activated form (Edkins et al. 2006; Tripathi and Garg 2018; Hobbs and Der 2019; Muñoz-Maldonado et al. 2019). Similar to this, loss of GAP activity can occur through deleterious mutations in GAP also. For instance, deletion of neurofibromin (NF1), a Ras GAP occurs in cancer frequently. The NF1 gene is highly susceptible to mutations forming loss of function mutants, which are responsible for sporadic cancers. The earliest case of NF1 mutations has been reported in recurrent glioblastomas. NF-1 also plays a major role in lung adenocarcinoma together with K-Ras mutations (32%), and EGFR mutations (11%). Moreover NF-1 is the third important mutated gene in melanoma after B-Raf and N-Ras (Ratner and Miller 2015). Other Ras GAPs such as RASA1 and RASA2 have been associated with breast and prostate cancer and drive invasion and metastasis (Sung et al. 2016; Suárez-Cabrera et al. 2017). Moreover, RAS mutations that inhibit GTP hydrolysis (such as F82V and T83P) or increase the activity of GEFs (S35T, A57G, and Y89H) have been identified in activation of Ras-related protein Rit1 and Rit2 in lung adenocarcinoma. The Rit proteins are involved in activation of various types of Ras effector molecules (Simanshu et al. 2017). Post-translational modifications such as prenylation and geranylgeranylation also play an important role in functioning of Ras proteins, specifically K-Ras and N-Ras (Adjei 2001).

Similar to Ras, mutations in Raf proteins are considered highly critical in induction of cancerous growth. Most of the research till date has been focused on Raf-1 and B-Raf. B-Raf mutations are widely described and responsible for causing tumor formation in melanoma (66%) and other malignancies such as glandular carcinoma (thyroid, ovary, biliary tract) (Rahman et al. 2013). The most predominant mutation detected is V600E that affects the kinase activity of B-Raf protein. Cancer-associated B-Raf mutations are found in exons 11 and 15 of the kinase domain. These mutations tend to form a gain of function mutant, which amplifies the downstream signaling constitutively, and thereby enhancing the growth and proliferation. Mutations also promote heterodimerization of B-Raf with Raf-1, thus activating the Raf-1-mediated ERK signaling (Poulikakos et al. 2011; Maloney et al. 2021). Several mouse models of melanoma have been studied to investigate the activity of B-Raf mutants. V600E mutants are generally responsible for development of benign and malignant melanomas. However, V600E exhibits formation of benign tumors at a higher frequency than malignant tumors. This has also been reported by conditional expression of V600E mutant in mouse model melanocytes (Matallanas et al. 2011). Mutations in B-Raf induce a senescence mechanism that regulates the dormancy and subdues tumorigenesis. In general, Raf and Ras mutations work independent of each other, however, several B-Raf mutations such as D594V have been reported which impair the kinase activity when coexpressed

with mutant Ras (Moretti et al. 2009; Roring and Brummer 2012). Raf-1 mutations associated with cancer are very rare and are reported in therapy-related acute myeloid leukemia. Besides mutations, rearrangement, and fusion of Raf-1 with B-Raf have been reported in thyroid cancer, prostate cancer, astrocytoma, and other cancers (Matallanas et al. 2011).

2.7.4 Therapeutic Targeting of Ras–Raf Interactions

The oncogenic Ras/Raf interactions are druggable, and hence potent small molecules have been discovered that bind either to Ras or Raf thereby inhibiting the oncogenic effect of Ras/Raf mutants. Targeting Ras-driven cancers is a little complicated, and the Ras–Raf interactions can be targeted by four ways: (1) scaffold-mediated targeting of Ras mutant, (2) targeting enzymes involved in post-translational modification of Ras proteins for inhibition of membrane association, (3) targeting Ras effectors, and (4) targeting MEK/ERK kinases (Santarpia et al. 2012; Papke and Der 2017; Degirmenci et al. 2020).

The most common drugs targeted in Ras-driven cancers are G12C K-Ras mutants. AMG510 and MRTX1257 are the primitive drugs developed to target K-Ras G12C mutations in lung cancer. Both drugs covalently bind to the switch II pocket and crosslink with Cys12. Scaffolding proteins are also known to inhibit mutant Ras functions on cancerous cells (Ni et al. 2019; Liu et al. 2019; Nussinov et al. 2021). Stevens et al. (2018) showed that Erbin, a LAP scaffolding protein functions as tumor suppressor in colorectal cancer. Erbin binds to the K-Ras kinase suppressor, and displaces K-Ras for Ras–-Raf complex inhibiting the downstream signaling (Stevens et al. 2018). Mutant Ras proteins are also targeted via inhibiting functionally relevant post-translational modifications. The farnesyltransferase (FT) is involved in prenylation of CaaX motif situated at the C-terminal of Ras proteins. FT and other transferases such as geranylgeranyl transferase (GGT), isoprenylcysteine carboxyl methyltransferase (ICMT) are important targets for designing inhibitors that indirectly target K- and N-Ras (Liu et al. 2019). The ICMT inhibition leading to inhibition of carcinogenesis has been widely studied in cell and mouse models (Xu et al. 2019). Another target for inhibition of Ras–Raf pathway is by targeting Ras effectors. Small molecule inhibitors such as Rigosertib, Sulindac, and MCP110 are inhibitors of Ras effectors. Raf kinases are the key targets of Ras mutants, thus making it a potent target for development of anticancer therapeutics (Quevedo et al. 2018).

A variety of inhibitors such as Vemurafenib, Dabrafenib have been applied for treatment of V600E B-Raf mutant-induced cancers. These drugs are known as first generation Raf inhibitors; although the efficacy of these drugs was found to be remarkable in clinical studies, with time the efficacy declined owing to the expeditious rate of drug resistance (Croce et al. 2019). Resistance to anticancer drugs is achieved by two mechanisms which reboots the ERK pathway upon treatment. Firstly, by upregulation of active Ras and ERK signaling via other Ras effectors or due to alternative splicing of B-Raf mutant specifically at N-terminus such that it

enhances the homodimerization reducing the drug affinity (Panda and Biswal 2019). Interestingly, resistant cancer cells utilize Raf inhibitors for their own survival. Hyperactive ERK signaling leads to cell senescence in normal or susceptible cancer cells. However, in drug-resistant cancer cells, these inhibitors monitor the activity of ERK signaling and maintain the optimum level required for cellular growth. A second generation of Raf inhibitors have been designed to overcome the drug-resistant issue. Some examples of second generation Raf inhibitors include pan-Raf inhibitors such as TAK632, CCt3833, Raf265, BAL3833, and paradox breakers such as PLX8349. The pan-Raf inhibitors inhibit both units of Raf dimers, while paradox breakers stimulate conformational changes in dimer to prevent dimer-dependent activation of MEKs. Moreover, MEKs and ERKs are also a potential target for inhibition of haywired ERK signal transduction (Yap et al. 2021; Degirmenci et al. 2020). Chemicals such as trametinib and cobimetinib are FDA-approved MEK inhibiting drugs for treatment of V600E B-Raf mutant-related cancers (Odogwu et al. 2018; Schmitt et al. 2022). However, usage of MEK/ERK inhibitors may not be a correct option for treatment of Ras/Raf-related cancers, as they can inhibit signaling pathways in normal cells also. Moreover, Rafs have been identified as more druggable targets compared to Ras proteins.

2.8 PKA Signalosome

Signalosomes are large multimeric protein complexes which increase the local concentration of each monomeric component for elevation of their signaling activity. Protein kinase A or PKA signalosome utilizes the GPCR-cAMP-PKA signaling axis for stimulation and maintenance of cellular functions. PKA is a cAMP (cyclic adenosine monophosphate)-dependent Ser/Thr kinase (Miller 2002). It is evolutionarily conserved in all eukaryotes and performs vital function in metabolic, developmental, and proliferative mechanisms. The PKA is an effector molecule utilized by secondary messenger, cAMP in cAMP-mediated GPCR signaling pathway. The activation and deactivation of PKA are regulated by cAMP-induced allosteric mechanisms. The PKA exists as a tetrameric holoenzyme in its inactive state. The tetramer consists of two regulatory and two catalytic subunits. The regulatory subunit is an auto-inhibitory domain which keeps the PKA in the inactive state. While the catalytic subunit mediates ATP hydrolysis in an active state for downstream signal transduction (Torres-Quesada et al. 2017). Four types of regulatory subunits—RIα, RIβ, RIIα, RIIβ, and three forms of catalytic subunits—Cα, Cβ, Cγ are expressed in cells. PKAs are classified into two types based on the regulatory subunits present; they include: PKA type I (RIα2C2, RIβ2C2), and PKA type II (RIIα2C2, RIIβ2C2). The cAMP-PKA signaling is driven by numerous ligand-receptor complexes of which the GPCR-cAMP-PKA signaling is a well-studied mechanism. Upon activation of adenylyl cyclase by GPCRs, cAMP is produced. Four cAMP molecules bind to the regulatory subunit of PKA to modulate structural changes and to release the catalytic subunit of PKA. The active PKA further phosphorylates the downstream effector protein. The activation of PKA induces

Table 2.1 Summary of the role of cAMP-PKA–effector signaling on different types of cancer

Cancer	Role of PKA	References
Chronic lymphocytic leukemia (CLL)	Stimulation of TLR signaling and apoptosis	Zhang et al. (2020)
Acute myeloid leukemia (AML)	Overexpression of CREB protein	Pigazzi et al. (2007), Illiano et al. (2020)
Breast cancer	Promotes growth and metastasis via GSK3-β-catenin pathway	Wang et al. (2019)
Prostate cancer	Upregulates AR signaling and neuroendocrine differentiation of prostate cancer	Merkle and Hoffmann (2011), Grigore et al. (2015)
Adenocarcinoma	Downregulation of SIRT1; inhibition of NSCLC cell apoptosis; upregulation of hypoxia-induced EMT	Farcas et al. (2019), Luo et al. (2019), Shaikh et al. (2012)
Brain tumor	Upregulation of p21/p27 and PKA/Epac-1-Rap1 signaling resulting in inhibition of glioblastoma	Wang et al. (2017b)

the PKA localization, which depends upon PKA-anchored proteins (AKAPs). AKAPs are scaffolding proteins that organize PKA macromolecular complexes for relaying pathway signals. A detailed information of the role of different PKA signalosomes has been reviewed elsewhere (Torres-Quesada et al. 2017; Rinaldi et al. 2018).

The GPCR-mediated PKA signalosomes are an important factor in several diseases such as cancer and degenerative diseases. Similar to Ras–Raf interactions, mutations in PKA subunits steer the PKA activity and its PKA-mediated signaling circuits. Being a nodal part of GPCR signaling, PKA has been widely studied in cancer, and is an accepted biomarker to cancer detection (Zhang et al. 2020; Caretta and Mucignat-Caretta 2011). The dysregulation of PKA and its effectors either boosts proliferation or induces apoptosis of the cell. Beside genetic alterations, localization of PKA subunits also affects functional properties of PKA. For instance, PKA type I are cytoplasmic proteins that are overexpressed in a variety of tumors. While PKA type II are anchored to organellar membranes and expressed in nonproliferative tissues in growth retardation phase. PKAs regulate cancer progression through phosphorylation of a variety of substrates such as CREB, Raf, CDC42 interacting protein 4 (CIP4), GSK3, calmodulin-dependent kinase (CaKs), etc. CREB, Raf, CaK, and GSK3 are directly involved in gene expression, survival, metabolism, and migration respectively. PKAs regulate the lipid metabolism with the help of epigenetic effectors such as JMJD3 and promote pancreatic cancer. Moreover, PKA also regulates actin polymerization via interaction with CIP4. CIP4 stimulates rearrangement of cytoskeletal proteins and cell membrane promoting the cancer metastasis (Stefan et al. 2018; Zhang et al. 2020). The cAMP-PKA-effector signaling acts as both tumor-suppressing and tumor-inducing factors. The effects of cAMP-PKA–effector signaling on different types of cancer are summarized in Table 2.1.

Targeting PKA signalosomes in cancer is a potential strategy for treatment of cancer. PKA signalosomes can be targeted by (1) inhibition of cAMP-PKA binding using cAMP analogs such as tacladesine, (2) targeting AKAP–PKA interactions, (3) inhibition of kinase activity, and (4) inhibition of PKA effectors (Sapio et al. 2014). Tacladesine has been investigated as an antitumor drug against colon cancer, breast cancer, leukemia, and other cancers by in vitro and in vivo experiments (Baiocchi et al. 2021). PKA and cAMPs also mediate a feedback regulation to control the cAMP-PKA signaling and increasing the levels of cAMPs can have a potent suppression of tumor growth. Hence, to maintain the concentrations of cAMP, several PDE (phosphodiesterase) inhibitors such as sildenafil have been studied for their role in tumor suppression (Peng et al. 2018). As discussed earlier, localization of PKA signalosome imparts a vital function in regulating the organellar specific roles of cAMP-PKA pathway. The localization of PKAs depends on their interaction with AKAPs. Hence small-molecule inhibitors such as peptides have been investigated for their antitumor activity. These peptides are derived from the PKA-binding domain of AKAPs which exhibits competitive inhibition of AKAP–PKA interaction, and thereby inhibiting the signal transduction. Few examples of AKAP-PKA inhibitors are HT31, RIAD, and STAD peptides (Dema et al. 2015; Bucko and Scott 2021). The kinase activity is normally regulated by the regulatory subunits. However, mutations in catalytic or regulatory subunits may lead to weak binding of both subunits. Antisense technology targeting PKA RIα is under investigation for development of therapeutic treatments. Herein, an antisense oligonucleotide, GEM231 has been investigated up to phase I clinical trials in patients with refractory tumors (Agrawal et al. 2002). Other than targeting cAMP and PKA itself, the downstream effectors of PKA have been studied as a potential drug target. Since CREB is the most common effector of cAMP-PKA signaling, CREB antagonists are under investigation and a small molecule—XX-650-23 exhibits proapoptotic and cell cycle arresting properties by blocking interaction between CREB and its coactivator CBP (Sapio et al. 2020). Although several drugs have been identified through preclinical studies; patients' trials are still undergoing for assessment of the ADME characteristics of PKA-associated antitumor drugs.

2.9 Myc–Max Interactions

Myc belongs to the family of basic HLH-leucine zipper motif containing (B-HLH-LZ) transcription factors. The Myc gene is a proto-oncogene and exhibits key roles in activation of cell cycle, differentiation, metabolism, and apoptosis. Three types of Myc proteins are expressed in cells: MYC (c-Myc), MYCL (l-Myc), and MYCN (n-Myc). Myc is a transcription factor, which consists of three characteristic motifs: a basic DNA-binding motif, helix-loop-helix (HLH), and a leucine zipper motif (Blackwood et al. 1992). Myc is activated by a variety of mitogenic signaling pathways such as Wnt, Shh, and EGF signaling pathways. Upon activation, Myc upregulates cyclins, ribosomal RNA, and cell cycle-related proteins for cell proliferation and growth, while downregulates p21 and

antiapoptotic proteins such as Bcl-2. It is generally expressed as an inactive intrinsically disordered protein which is localized in cytoplasm. The inactive Myc is cleaved by calpain, a proteolytic enzyme to produce c-Myc and n-Myc. While Max or Myc-associated factor X is another HLH-based leucine zipper transcription factor that interacts with Myc. The Myc-Max heterodimer binds to the E-box of the gene promoter region with the help of chromatin-modifying complex (TRRAP, TIP60, and GCN5), and induces gene transcription. Max interacts with the C-terminal B-HLH-LZ domain and drives transcription by heterodimerization (Cascón and Robledo 2012). Besides upregulation of cell cycle, antiapoptotic, and proliferation-related genes; Myc–Max complexes are also key regulators of miRNA expression and metabolism-regulating genes. Owing to the functional characteristics of Myc–Max interactions, it has been extensively studied in cancer prognosis (Chen et al. 2018a). Of all the Myc proteins, c-Myc is often constitutively expressed in cancers. Although expression of Myc is normally transient and highly controlled by ubiquitin ligases (Ub-ligases), chromosomal translocations and mutations lead to dysregulation of Ub-ligase-Myc interaction and activation of tumorigenesis. Moreover, more than 70% of human cancers such as retinoblastoma, breast cancer, ovarian cancer are associated with overexpression of Myc–Max complexes (Dang 2012).

Inhibition of Myc protein is a difficult task due to intrinsic difficulties such as nonavailability of catalytic sites. Hence, targeting Myc–Max interactions can play a pivotal role in regression of cancer. The indirect inhibition of Myc protein is targeted by: (1) disrupting Myc transcription, (2) inhibiting Myc mRNA translation, (3) targeting Myc stability, and (4) destabilizing Myc–Max complex. The transcription of Myc gene is regulated by bromodomain-containing 4 (BRD4) and cyclin-dependent kinases (CDK7 and CDK9). BRD4 belongs to the BET family of transcription factors and over the years, a wide variety of BET inhibitors have been investigated for their anticancer activity (Xu and Vakoc 2017). The first BET inhibitor identified was JQ-1, which binds to the acetyl-binding domain of BRD4 important for chromatin attachment. Similarly, I-BET762 has been identified as BET inhibitor and has undergone initial phases of clinical trials in patients with hematologic malignancies and NUT Midline carcinoma (Pérez-Salvia and Esteller 2017). CDKs also stimulate myc gene transcription; hence, inhibitors of CDKs are also utilized for inhibition of myc transcription. Myc protein is stabilized by several deubiquitinating enzymes such as USP7 and USP28. USP inhibitors such as P22077 have been studied as a suppressor of neuroblastoma in xenograft models (Young et al. 2019). Several small molecules such as Mycmycins, Mycro 1–3, and KJ-9 have been elucidated as antagonists of Myc–Max interactions. KJ-9 and KJ-10 are derivatives of Krohnke pyridine derivatives that have been shown to inhibit Myc-Max dimerization and Myc-induced tumor growth in vivo (Hart et al. 2014; Fletcher and Prochownik 2015). Similarly, Choi et al. identified another small molecule—sAJM589 as a potent Myc-Max antagonist using protein-fragment complementation assay (PCA) (Choi et al. 2017). The treatment of Myc-driven cancer is also targeted by inhibition of enzymes involved in Myc-regulated metabolic enzymes. Myc upregulates expression of several glycolytic and glutaminolytic

enzymes and transporters. Recently, a novel inhibitor L755507 has been identified that efficiently blocked Myc–Max interactions together with induction of apoptosis in cancer cells (Singh et al. 2021). Targeting inhibition of Myc-regulated genes using miRNAs and small inhibitors also offers a bright opportunity as anticancer therapeutics.

2.10 p53–MDM2 Interactions

p53 is a tumor suppressor cytoplasmic protein. It inhibits cell growth and mediates senescence, apoptosis, and responses to the cellular damage (Selivanova 2004). A variety of stress types induce expression of p53 protein thereby preventing the uncontrolled propagation of cells. Moreover, p53 partakes in a dual transcription-dependent/independent function and destroys the cell. It regulates transcription in the nucleus as well as mitochondria (Thut et al. 1997; Moll et al. 2005). In a healthy cell, the concentration of p53 is low, as it is highly unstable with a half-life ranging from 5 to 30 min. p53 degradation in a normal cell is mediated by MDM21. However, in a damaged cell, the DNA damage, hypoxia, and oncogene activation stimulate rapid stabilization of p53 through blocking the p53 degradation pathway thus leading to cancer progression. MDM2 is a principal cellular antagonist of p53 (Michael and Oren 2003). It is an E3 ubiquitin ligase, and a negative regulator of p53 tumor suppressor. MDM2 recognizes the transactivation domain of p53 and inhibits transcriptional activation. p53 and MDM2 are linked with each other through an autoregulatory negative feedback loop (Konopleva et al. 2020). This limits the duration and severity of p53-associated biological function after a stress response. MDM2 promotes degradation of p53 via a ubiquitin-dependent pathway using both nuclear and cytoplasmic 26S proteasomes.

The p53–MDM2 interactions have been mapped through genetic and biochemical studies (Åberg et al. 2017). The N-terminal domain of MDM2 and the N-terminal part of TAD of p53 interact with each other. The crystal structures of p53-MDM2 complex have revealed that upon binding with MDM2, the unstructured p53 TAD forms an amphipathic alpha helix where hydrophobic residues F19, W23, and L26 are buried into deep hydrophobic pocket on the MDM2 surface. The structural changes in p53 are relevant for its activity in transcription (Raj and Attardi 2017). As the three hydrophobic residues F19, W23, and L26 are important for interaction of p53 and MDM2, several peptides have been designed and studied using combinatorial permutation and biophysical studies to understand the structural requirements of p53–MDM2 interaction. Moreover, various highly potent peptidomimetic antagonists of MDM2 have been designed using drug design and NMR spectroscopy in the recent years. One such example is an AP peptide (Zhang et al. 2015). Due to the great importance of p53–MDM2 interaction in cancer, it is often used as a drug target by targeting the interaction of three critical amino acid residues as mentioned above (Wang et al. 2017c). A detailed discussion on the various p53-MDM2 drug targets and their mechanism of action is discussed in Chap. 7 of this book.

2.11 Wnt/β-Catenin

Wnt/β-catenin PPI is a well-maintained signaling interface involved in conventional biological phenomenon including proliferation, differentiation, cell death, cellular migration, and homeostasis. Accumulated evidences indicated that the impairment in Wnt/β-catenin signaling intend toward the growth and advancement of tumors and chronic cases of malignancies; it is well studied in the case of colorectal cancer (He and Tang 2020; Gajos-Michniewicz and Czyz 2020). As an outcome of elaborative studies conducted on Wnt/β-catenin interface, it is unraveled that β-catenin employs Kat3 transcriptional coactivators, CBP and its homologs, and p300 (E1A-binding protein, 300 kDa) to generate a complex that is transcriptionally active and regulate the transcription and expression of target genes responsible for the growth and differentiation of the cells. A transcriptional program mediated by p300/β-catenin interplay activates the differentiation and reduces its cellular potency (the cell's potential to differentiate into some other cell types), whereas transcription mediated by CBP/β-catenin is imperative for maintaining stem cell/progenitor cell and symmetric nondifferentiative division (Thomas and Kahn 2016). Atypical regulation of transcription factor β-catenin is evident in the primary events of carcinogenesis, as it initiates the uncontrolled expression of target genes facilitating the unrestricted differentiation of cells (Fig. 2.5). The β-catenin-dependent signaling

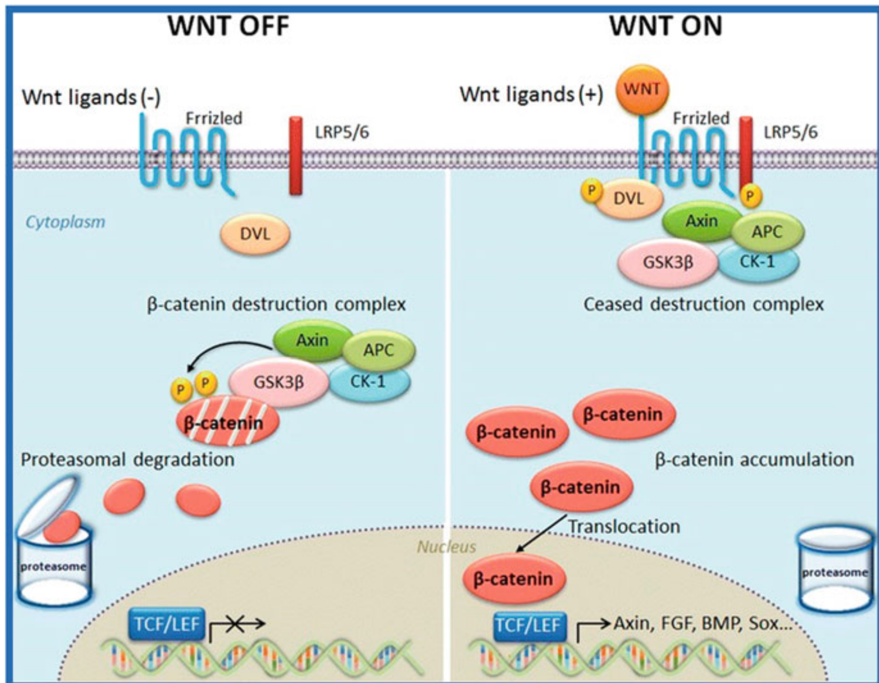

Fig. 2.5 Summarized representation of WNT/β-catenin signaling. (Adapted from Ota et al. 2016)

pathway is activated when secreted glycoprotein Wnt ligands that are rich in cysteine residues bind to LRP-5/6 and FZD receptors, which leads to the agglomeration of destruction complex comprises of AXIN, GSK3β, CK1, and APC on the receptor (Nusse and Clevers 2017). Following that, phosphorylation, and suppression of GSK3 result in an increase in cytosolic β-catenin levels. Unphosphorylated β-catenin in the cytoplasm relocates to the nucleus and interacts with TCF/LEF, their coactivators such as Pygopus and Bcl-9, and stimulates expression of Wnt signaling-associated genes such as c-Myc, cyclin D1, and CDKN1A (Wiese et al. 2018).

Considering Wnt/β-catenin significance in cancer induction and progression, various molecules (antagonist, inhibitors agonist) targeting this interface have been proposed and investigated. In line with this, Ipafricept (OMP54F28; IPA), an antagonist of Wnt ligands that competitively interacts with Frizzled and other Wnt-associated receptors and inhibits Wnt-arbitrated downstream processes (Moore et al. 2019). On the similar note, pyrvinium, is an FDA-approved drug which hampers the nuclear localization of the β-catenin by stabilizing the β-catenin destruction complex; this drug binds to the CK1 component of the complex and potentiates its kinase activity (Thorne et al. 2010). Moreover, Wnt/β-catenin axis may crosstalk with various other signaling proteins and leads to the numerous PPIs, thus contributing to the crucial molecular mechanisms in cancer induction and its advancement. These proteins include, dermal growth factor receptor (EGFR), Hippo/YAP, NF-Kb, PI3K/Akt pathway, Sonic Hedgehog pathway, and Notch signaling (Zhang and Wang 2020). As a result, PPI inhibitor targeting the cross talk between Wnt/β-catenin axis and aforementioned proteins is under scrutiny, and shown to have therapeutic potential in preclinical investigations and clinical trials of several cancer types (Joosten et al. 2020).

2.12 Nuclear Receptor (NR)

Nuclear receptors (NRs) are a ligand-activated transcription factor superfamily that takes up critical roles in the pathophysiology of many physiological processes including development, metabolism, reproduction, aging, and disease conditions like cancer. NRs are an essential platform that links external and hormonal cues with genomic responses, and controls almost all types of cellular fate at the gene expression level (Zhao et al. 2019). Several NRs have long been recognized for their critical involvement in cancer genesis and progression as summarized by Weikum and coworkers (2018). The biological effects of NRs in managing proliferation, differentiation, and apoptosis signify their potential impact on tumor growth. Indeed, some medications that target the interface of NRs-ligands have been the subject of considerable drug discovery attempt for effective cancer therapies (Font-Díaz et al. 2021). The known 48 NRs are majorly divided in two subfamilies depending upon the location and genomic response to ligands as cytoplasm-based and nuclear-based NRs. Cytoplasm-based NRs comprise of androgen receptor, estrogen receptor, and glucocorticoid receptor (AR/ER/GR). In this case, ligand binding promotes the

release of chaperone and induces homodimerization, which lead to nuclear translocation. Once in the nucleus, the ligand-bound receptor connects with transcriptional coregulators, that allow their interaction with the transcriptional machinery to initiate regulation of target gene expression. Meanwhile, nucleus-based NRs include vitamin-based receptors such as VDR, RAR (Vitamin-D receptor and Vitamin-A1 (retinoic acid) receptor), liver X or peroxisome-proliferator-activated receptors (PPARs and LXRs). In the absence of ligand, they generally dwell in the nucleus and are associated with their corresponding DNA sequences (Sever and Glass 2013).

The proteins of NR superfamily are distinguished by the fact that, they serve as transcription factors. The disruption of transcriptional regulation or the loss of function of these receptors is a defining feature of many cancers. The contribution of androgen receptor (AR) involvement is well reported in the prostate cancer, as prostate is an androgen-dependent organ, where AR overexpression on the cells enables the prostate cancer to develop (Shafi et al. 2013). Interaction of AR with proteins like HSPs, and cyclin-dependent kinase 5 (Cdk5) are responsible for its regulation; these PPIs were explored as the potential target for the inhibitors to regulate the progression of cancer. Ailanthone, a small molecule PPI inhibitor demonstrated to act on the AR/HSP90 interface by hampering the translocation of AR to the nucleus, and in turn hinders the interaction with HSP90 (He et al. 2016). On the similar note, roscovitine was reported to inhibit the interaction between AR/Cdk5 to reduce the proliferation of prostate cancer (Hsu et al. 2011). In addition to AR, estrogen receptor (ER) that is crucial for estrogen-mediated signaling is predominantly involved in the development of breast and uterus cancer. ER receptor is reported to encourage the cancer development either through dimerization (homo/heterodimerization) and/or PPI with other protein regulator (Miki et al. 2018). As reviewed by Zhao et al., currently numerous therapies targeting the PPI involving ER are in practice that work either through disruption of estrogen production or by influencing the activity of ER in breast cancer cells (Zhao et al. 2019). Small-molecule PPI inhibitor like tetrahydro-iso-alpha acid and one of the derivatives of benzothiophenone is demonstrated to hinder the interaction of ER with its cofactor protein (Miki et al. 2018). Furthermore, protein–protein interactions in peroxisomes proliferator-activated receptor (PPAR) are also a potential therapeutic target, owing to the fact that PPARs are crucially involved in the apoptosis. The three members of this PPAR family (PPAR $\alpha/\delta/\gamma$), interact with retinoid X receptors (RXRs) and form a heterodimer that further triggers the tumorigenic cascade (Wang et al. 2017a). Likewise, other NRs, like the glucocorticoid (GC) receptor (GR), retinoic acid receptors (RARs), and retinoid X receptors (RXRs), have been actively researched as therapeutic targets in cancer, with some resulting in additional marketed medications as described in a review by Zhao et al. (2019).

2.13 X-Linked Inhibitor of Apoptosis Protein (XIAP)/Caspase-9

Inhibitors of apoptosis proteins (IAPs) are a category of ubiquitous protein comprising of antiapoptotic properties that are imperative to cell survival. Due to their capability to attach to caspases or other proteins, they are able to inhibit the actions of the proapoptotic proteins and encourage their destruction, which helps in regulating the process of apoptosis. There are variety of other activities performed by IAPs in nonapoptotic pathways that contribute to phenomenon like migration, invasion, and metastasis (Rumble and Duckett 2008). XIAP, c-IAP1, c-IAP2, ML-IAP/Livin, ILP2, NAIP, Bruce/Apollon, and survivin are the members of the IAPs family. Furthermore, all these members of IAPs include baculoviral IAP repeats (BIR) (1–3 BIR repeats), which function as protein–protein interaction domains. Caspase, a proteolytic enzyme having cysteine residue is one of the primary facilitators of apoptosis and interactors of IAPs. Caspases are reported to induce apoptosis through two pathways; (a) the death receptor route (extrinsic pathway) administered by caspase-8 and 10; (b) mitochondrial (intrinsic) pathway, which is coordinated by cytochrome C/caspase-9 (Fig. 2.6) (Tu and Costa 2020).

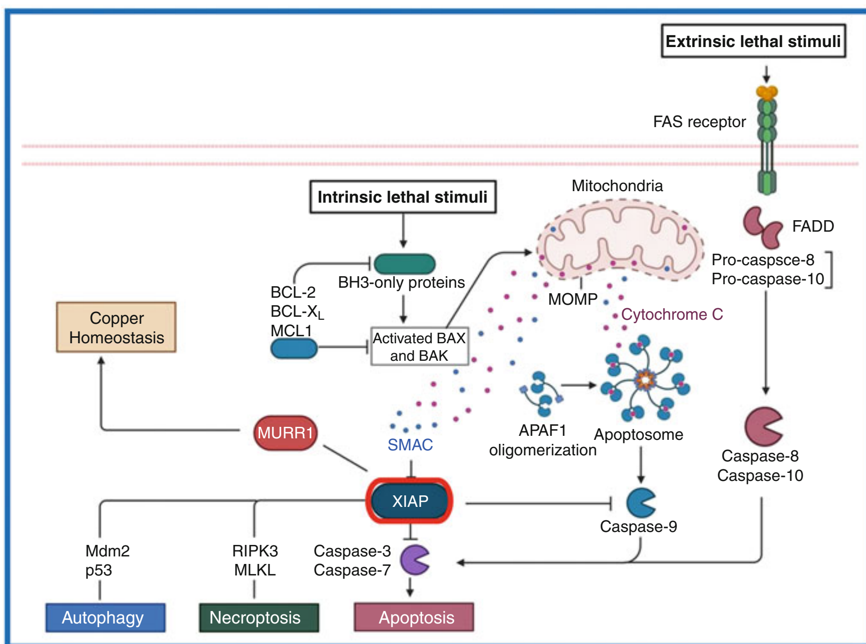

Fig. 2.6 Schematic representing the biological functions performed by XIAP protein. XIAP primarily inhibits caspases 3/7/9 by binding, hence blocking preapoptotic signaling cascades. SMAC, a mitochondrial protein, prevents XIAP from performing its activities by consuming its binding positions. RIPK3 and mixed lineage kinase domain-like pseudo kinase (MLKL) transition XIAP to necroptosis, Mdm2 and p53 induce autophagy, and MURR1 modulates the homeostasis of copper. (Adapted from Tu and Costa 2020)

Caspases are regulated by apoptosis inhibitors (IAP) proteins in live cells; XIAP is the only protein member of IAP family that suppresses caspases by direct physical contact. Other members of the IAP family that includes cIAP1 and cIAP2 are also reported to bind caspase-3 and -7 respectively; and marks them for proteasomal degradation rather than blocking them physically. The BIR3 domain exhibits high affinity toward caspase-9 and the interaction between them acts as inhibitory signal against the proapoptotic functions of the caspase-9 and stops the programmed cell death (Salvesen and Duckett 2002). Apparently, Smac (second mitochondria-acquired activator of caspase) is a domestic PPI inhibitor that is reported to suppress the XIAP–caspase-9 relationship. As Smac is discharged from the mitochondrial region, the N-terminal BIR3-binding motif (AVPI) of Smac interacts with the BIR3 repeats, and prohibits the interaction of XIAP with caspase and thereby promoting apoptosis (Abbas and Larisch 2020).

XIAP is abnormally expressed in a number of cancers/tumors in human and promotes chemotherapeutic treatment resistance in particular patient subgroups (Obexer and Ausserlechner 2014). The therapeutic efficacy of XIAP inhibition in cancer therapy has sparked interest in describing machineries that control XIAP availability such that the inhibitors impeding the XIAP activities can be investigated. In recent studies, Smac protein mimetic has been used to suppress the activity of XIAP/caspase9 complex. GDC-0152 was the first Smac mimetic discovered using the combinatorial approach of peptidomimetic techniques and HTS (high-throughput screening) that matches the structure of the Smac and binds to the BIR domain of XIAP with great specificity and inhibits the PPI between XIAP/caspase-9 (Flygare et al. 2012). On the similar note, GDC-0917 (CUDC-427), an another Smac mimetic has entered phase I clinical studies for the safety assessment of patients with locally advanced tumor and lymphomas (Tolcher et al. 2016). Recently, Novartis LCL-16, a small molecule PPI inhibitor hindering the association of XIAP/caspase-9 has also gained much attention for showing positive results against the triple negative breast cancer and is in the second phase of clinical trials (Bardia et al. 2018).

2.14 HSP90/Cdc37

Whenever a viable three-dimensional structure of a protein is not satisfied, chaperone-mediated aggregation or degradation of proteins occurs. Heat-shock proteins (HSPs) are the groups of molecular chaperones which are activated under stress to prevent protein denaturation and improper aggregation in turn to preserve protein homeostasis. Under nonstress situations, HSPs are imperative to a variety of cell maintenance processes like proliferation, apoptosis, and protein trafficking (Edkins et al. 2018). HSP90, a 90 kDa heat shock protein, is among the most prevalent and well-conserved chaperones, comprising for 1–2% among all cellular proteins, and rising up to tenfold in response to physiological stress. At present, four isoforms of the HSP90 have been recognized that include cytoplasmic isoforms (HSP90α and HSP90β), ER isoform (GRP94), and mitochondrial isoform (TNF

receptor-associated protein 1; TRAP1) (Butler et al. 2015). HSP90 is a homodimer composed of three evolutionarily conserved domains: a N-terminal ATP-binding region, a central domain, and a dimerization region located at C-terminal. The ATP-binding motif is pivotal in hydrolyzing the ATP. The N-terminal governs the assembly of multimolecular chaperone complexes (having HSP90 as one of the constituents) by acting as a conformationally active transition area. The central domains behave as nuclear localization sequences as well as binding sites of the target protein. The domains in the middle differentiate distinct substrate proteins and govern the action of certain molecular chaperone substrates. The C-terminal motif of HSP90 is a self-dimerization site that ameliorates the independent action of two HSP90 N-terminal domains (Patel et al. 2011).

HSP90 expressions in tumor cells were found to be ~10 times greater than in normal cells, indicating that it is essential for the multiplication and survival of tumor cells. HSP90 also contributes in the development of protein kinases and transcription factors like Her2, VEGF, mutant p53, CDK4, HIF-1, Raf-1, and Akt, which influence the growth and apoptotic signaling cascades of cancerous cells (Birbo et al. 2021). Along with the abovementioned client proteins, HSP90 has also been linked to the stability and activation of over 300 other client proteins. HSP90 provides the stability to the structure of client proteins, and ubiquitination-mediated destruction of these client proteins is restricted, allowing them to remain in the active mode and facilitate tumor development and metastasis. However, targeting the HSP90-client protein interface may induce the degradation of client proteins that will promote the restriction of growth and progression of tumor (Calderwood and Neckers 2016). Based upon the structure of Hsp90, its inhibitors are majorly categorized into three subfamilies: ATP-binding cavity inhibitors, nucleotide-binding site inhibitors, and HSP90 and chaperone complex inhibitors. HSP90 regulates their molecular protein partners by affecting their ability to use ATP, as a result, the suppression of such a critical function has a significant toxicity, as it impacts numerous normal proteins (Garg et al. 2016); owing to this, Pfizer's HSP90 inhibitor SNX-5422 was withdrawn from phase 1 clinical study in 2011 owing to eye toxicity (Rajan et al. 2011).

Cell division cycle protein 37 (Cdc37) has received considerable interest among the various molecular partners of HSP90. Several oncogenic protein kinases (EGFR, CDK, and Akt) interaction with HSP90 and the spatial configuration of HSP90-kinase complex are dependent on Cdc37. Hence, targeting the HSP90-Cdc37 complex can deactivate the oncogenic kinases and hamper cancer cell proliferation and growth (Taipale et al. 2012). The structural data of the HSP90-Cdc37 based on X-ray and NMR experiments also highlight the targeted approach for designing the inhibitor for HSP90/Cdc37 protein complex. Though many inhibitors for HSP90/Cdc37 have been reported recently, an inhibitor known as DCZ3112 blocking the association of HSP90/Cdc37 has been reported. DCZ3112 specifically interacts with the HSP90 amino-terminal domain and suppresses the HSP90/Cdc37 communication such that it does not alter the HSP90 ATPase activity (Chen et al. 2018b).

2.15 Bax/Bcl-2

The Bcl-2 or B-cell lymphoma-2 family comprises about 20 Bcl-2-like proteins and is a critical regulator of mitochondrial apoptosis. The proteins of Bcl-2 family could be well categorized into two groups based upon their activity in apoptosis: (a) antiapoptotic proteins (Bcl/Mcl family proteins—like Bcl 2/w/A1, and Mcl-1); (b) proapoptotic proteins (such as Bcl-associated/interacting proteins such as Bak, Bax, Bok, Hrk, Noxa, and Puma) (Fig. 2.7) (Campbell and Tait 2018). The antiapoptotic and proapoptotic factors have the propensity to form dimers, which act as an apoptotic switch. Proapoptotic proteins like Bax and Bad play important part in the process of programmed cell death. When these proapoptotic proteins attach to antiapoptotic proteins such as Bcl-2, their actions are inhibited. Cancerous cells can be prohibited from evading apoptosis by hindering the association between pro- and antiapoptotic proteins (Coultas and Strasser 2003; Li et al. 2018). Almost all the proteins falling under Bcl-2 family are homologous, they include one or more conserved Bcl-2 homology (BH) motifs known as BH1, BH2, BH3, and BH4; Bid, Bad Bmf, Noxa, Puma, and Hrk, the well-known members of BH3. Bcl-2 has two hydrophobic α-helix structures surrounded by 6–7 amphiphilic α-helix structures, four of which create a hydrophobic BH3 "pocket" to bind with Bax (Petros et al. 2004). The Bcl-2/Bax homodimer exhibits better stability than the Bax/Bak homodimer, which reduces the function of Bax/Bak in initiating cell death, and inhibits the apoptosis of cells (Fig. 2.7) (Moldoveanu et al. 2014).

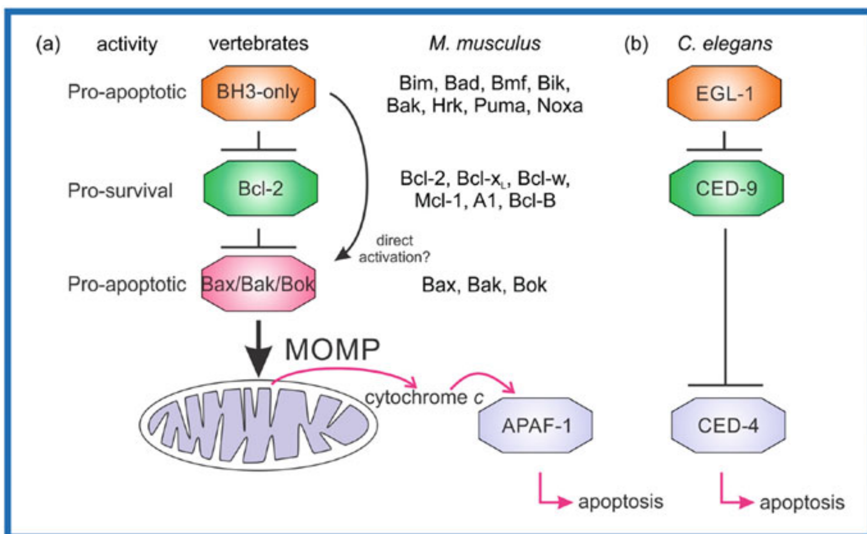

Fig. 2.7 Schematic representation of Bcl-2-mediated mechanism of apoptosis. Concise apoptotic schemes demonstrating the function of Bcl-2 proteins in apoptosis initiation in humans (**a**) and worms (**b**). (Adapted from Banjara et al. 2020)

The expression of antiapoptotic proteins such as Bcl-2 is upregulated in vast majority of human malignancies (majorly in prostate cancer), and seems to exhibit significant resistance to apoptosis, leading to cancer development (Li et al. 2018). Based on the involvement in tumor growth, hindering the function of Bcl-2 protein family affiliates can be a unique and potential therapeutic method for cancer prevention. Though, Bcl-2 protein family members have long been regarded as therapeutic targets, many of the proposed Bcl-2 inhibitors are nonspecific, impacting other cellular constituents. Owing to their nonmechanism-based toxicity, the applicability is limited. The proposed inhibitor of PPI between anti- and proapoptotic class of protein must be a mimic of proapoptotic protein possessing the greater affinity toward the antiapoptotic proteins and induce the apoptosis in cancerous cells (Billard 2012). Oltersdorf and coworkers have introduced a small molecule called ABT-737 that interacts with the BCL-X_L; this molecule was the first therapeutic wonder shown efficient inhibition against the cells of lung cancer and leukemia's. However, due to its insufficient absorption, the therapeutic application of ABT-737 was limited (Oltersdorf et al. 2005). Similarly, based upon the NMR-based SAR of ABT-737, a structurally modified molecule named ABT-263 was studied. In spite of showing inhibitory effect on the Bcl-2-Bax axis, it was not explored further due to its detrimental effect on the platelets counts of the patients (Lu et al. 2020). Further, taking the structural design of ABT-263 in the consideration; ABT-199, a molecule inhibiting the PPI of Bcl2-Bax was proposed that was having the higher binding affinity toward the Bcl-2 and showed tremendous effects against cancerous cells. In recent years, ABT-199 is the very first anticancer small molecule PPI inhibitor approved for the marketing (Lu et al. 2020). On the similar note, various strategies focusing on Bcl-2 family-associated protein–protein interactions have been comprehensively reviewed elsewhere (Edlich 2018; Matos et al. 2020), and will be discussed in the later chapter(s).

2.16 Splicing Factor 3b (SF3B)

Splicing of pre-mRNA into mature mRNAs by removing the intronic regions is an important criterion in the creation of high-fidelity transcripts and appropriately encoded proteins. Spliceosome, a complex composed of five small nuclear RNAs (snRNAs) that interacts with proteins to create elements known as small nuclear ribonucleoproteins (snRNPs), and catalyzes the whole process of pre-mRNA splicing. Till now, two categories of spliceosomes with distinct compositions have been identified—Spliceosomes that are U2-dependent (major), and U12-dependent (minor) (Sun 2020). The variations in the mechanisms of pre-mRNA splicing arbitrated by U2- and U12-mediated spliceosomes seem to affect initial procedures of intron recognition, but not the catalytic process. SF3b is a multiprotein U2 snRNP component that is required for pre-mRNA splicing. Human SF3b is a stable complex with seven subunits; this protein intermingles with the pre-mRNA at/near the branching site (BS) in the spliceosome, enhancing the U2 snRNA/BS base-pairing

interaction. As a result, they play an important part in BS detection and selection especially with constitutive and alternative splicing (Cretu et al. 2016).

The biggest subunit of the SF3B complex, SF3B1 (splicing factor 3b subunit 1), operates as a major component of the U2 snRNP, that is essential for branch site identification and the early stages of spliceosome formation (Will and Lührmann 2011). N-terminal domain of SF3B1 has several U2AF2-binding motifs. These motifs can probably aid in the positioning of the U2 snRNP to the branch region. Approximately, 75% of the C-terminus is made up of 22 nonidentical HEAT (Huntingtin, elongation factor 3, subunit of protein phosphatase 2A, and PI3K target of rapamycin 1) repeats that produce helical assemblies (rod-like), and serve as important framework for the U2 snRNP to facilitate the association with other SF3B components. Interestingly, most of the SF3B1 mutations reported in cancer are found in the HEAT domain (Alsafadi et al. 2016).

In perspective of SF3B1 as one of the fundamental constituents of splicing procedure, it is the pivotal component for the oligomerization of all the seven components of SF3B. It has been convincingly shown that SF3B1 mutations induce both common and tumor-specific splicing abnormalities. Mutated SF3B1 causes dysregulation of various cellular activities, such as heme biosynthesis, permeation of immune cells, response to DNA damage, construction of R-loop, telomere preservation, Notch signaling cascade, and many cellular cascades, including the mitochondrial, and NF-κB pathways, in many cancer cells. These outcomes advocated for the fact that any changes in the SF3B1 gene induce various functions in tumor growth (Sun 2020).

Interestingly, the gene of SF3B1 is one of the frequently altered in spliceosomal subunit as discussed in the case of breast cancer (Banerji et al. 2012), prostate cancer (Armenia et al. 2018), and the mutations in this gene are also strongly linked to ER-positive illness. It is interesting to see that, the inhibition of SF3B1 significantly reduces the number of protein–protein interactions, thereby equally reduces the aggressiveness of cancer cells via direct and indirect machineries, presumably including the control of several kinds of cellular stress mechanisms. Owing to this fact, inhibitors like E7107E and SSA are now in development and may have superior anticancer effect against malignant cells of prostate cancer (Obeng et al. 2016). The prognostic and therapeutic prospects of SF3B1 in various forms of cancer need to be investigated further.

2.17 MLL1-WDR5/Menin

Mixed lineage leukemia 1 (MLL1) is a crucial transcription factor and also a histone–H_3 lysine–4 (H3K4) methyltransferase; this protein is responsible for the regulation of hematopoiesis and embryonic growth through transcription of Hox genes. MLL1 is multisubunit protein of 3696 amino acids. N-terminal of MLL1 is approximately 1400 amino acid long that serves the purpose of transcription factor, whereas the C-terminal domain is acting as a H3K4 methyltransferase (Li and Song 2021). MLL1 and its key fusion partners are involved in several PPIs, that are

important in controlling the gene expression in conventional physiological condition as well as in induction and maintenance of leukemia (Xu et al. 2016). Despite their considerable variety, protein fusions seem to activate MLL through two distinct mechanisms: imparting inherent transcriptional effector activity or generating forced MLL dimerization and oligomerization. Both processes result in the abnormal expression of a selection of Hox genes, most notably HoxA9, whose persistent expression is distinguishable, but not a unique hallmark of human MLL leukemias (Milne et al. 2010). In MLL1 leukemia, chromosomal translocation results in the production of a fusion protein of oncogenic traits that composed of the MLL1 N-terminal DNA-binding domains (residues 1–1400) attached with one or more than 70 fusion partner proteins.

In humans, *wdr5* gene encodes for a protein called WD repeat-containing protein 5. This protein comprises seven WD repeats and is established to be interacting with host cell facet C1 and MLL. It is acting as the presenter of H3K4 methyltransferase domain of MLL protein. WDR5 is a crucial element that determines the MYC employment to chromatin (Wu and Shu 2011). Owing to the fact of being involved in the crucial cellular processes, MLL1-WDR5 axis has been explored as a significant target for therapeutic purpose. For instance, highly specific peptidomimetics have been investigated to regulate the WDR5 and MLL interaction, and it was observed to be having the alienating impact. Dysregulation in MLL is reported in number of leukemia, and disassociation of MLL1-WDR5 complex using peptidomimetics has been reported to diminish the occurrence of MLL-fusion arbitrated leukemogenesis (Karatas et al. 2013). Along with the peptidomimetics inhibitors, some nonpeptidomimetics inhibitors of MLL–WDR5 interface are also being explored as a potential anticancer agent (Ye et al. 2020).

Menin is a universally expressed nuclear protein that participates in a complex series of interactions (Fig. 2.8). Functionally, Menin is best established as a cofactor component of MLL1 and MLL2 complexes that sustain gene expression by methylation of H3K4 (Guru et al. 1998). The biochemical and structural study of the MLL-Menin PPI showed that the N-terminal of MLL1 attaches to a relatively wide central cavity on Menin, suggesting that small molecules cannot obstruct the whole MLL-binding site. Structural investigations indicated that the small region of the central cavity of Menin where MBM1 domain of MLL attaches (approx. 6–7 amino acid long) might be the most promising "druggable" target for inhibitor development (White et al. 2008). Various small-molecule antagonists of the menin–MBM1 interaction have been discovered till now. Menin engages a peptide fragment of MLL, and use of peptide-based inhibitor could be an appealing technique for targeting the MLL–Menin interaction. Structure-based development has recently resulted in the development of powerful peptidomimetics imitating the MBM1 fragment of MLL. Similarly, studies performed on nonpeptidomimetics also paved the path for the establishment of series of therapeutically active compounds, as summarized by Cierpicki and Grembecka (2014).

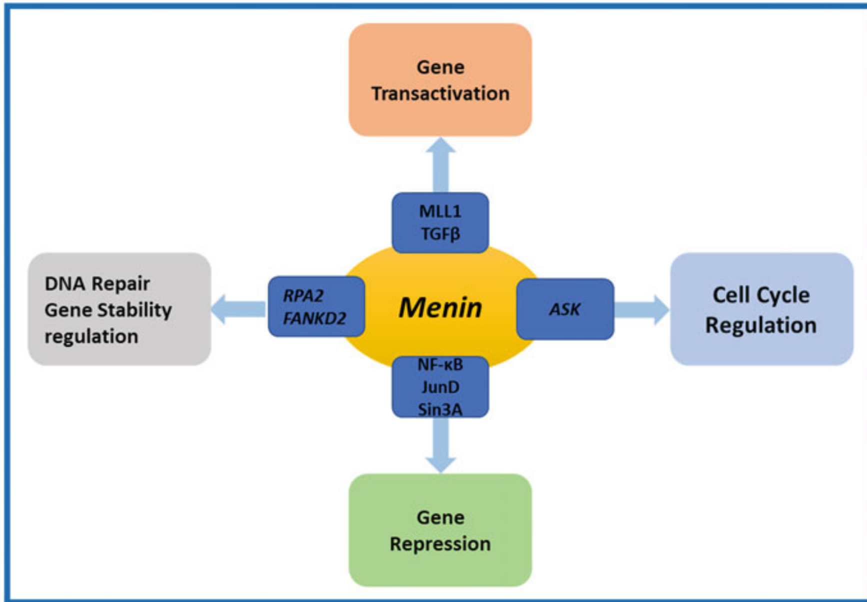

Fig. 2.8 Major interacting protein partners of Menin and their functional roles

2.18 Alpha/Beta Tubulin

The cell cytoskeleton is made up of microtubules, microfilaments, and intermediate filaments. The microtubule network has significant role in controlling the growth and mobility of the cells widely, as well as important signaling events that affect basic activities of the cell. Microtubules are made up of α- and β-tubulin heterodimers, which join together to develop a hollow heterodimeric cylindrical structure that actively extends, and shortens during the cell cycle (Roostalu and Surrey 2017). Motor proteins move the components of cellular machineries along the microtubule tracks, which are coordinated by protein–protein interactions among adaptor proteins. In cells, tubulin heterodimers are present in soluble form, and protein–protein interactions with all of these tubulin members influence the activity of microtubules (Nogales 2001).

Microtubules assemble to form spindles in living cells during the initial phases of cell division. During mitosis, chromosomes are pulled by the spindles toward the two poles, thereby resulting in two daughter cells caused by the division of chromosomes, and completion of cell proliferation. Under physiological circumstances, the microtubule and tubulin dimer maintain a dynamic equilibrium (Muroyama and Lechler 2017). Microtubules are made up of eight α-tubulin and seven β-tubulin isotypes having variable tissue distributions (Fig. 2.9). Tubulin family members share structural similarity and are differentiated substantially by

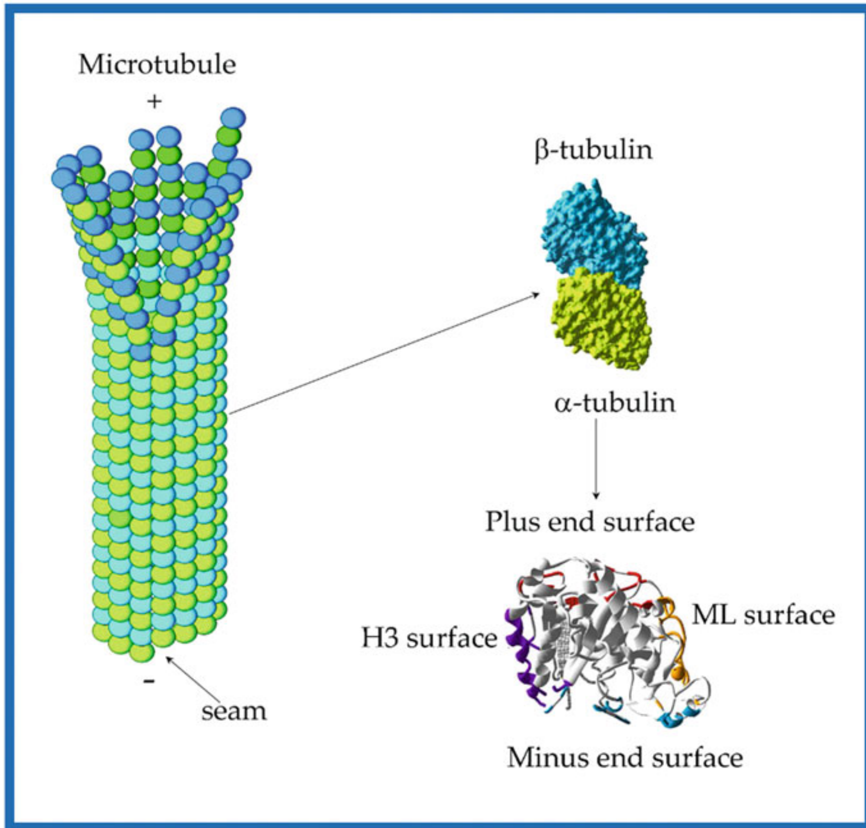

Fig. 2.9 Diagrammatic representation of structural component of microtubules. (Adapted from Borys et al. 2020)

varied sequences at the C-terminal tail. Tubulin's C-terminal tails are also hypothesized to facilitate the interactions between proteins, and serve as locations of post-translational modifications that are responsible for the varied activity of each isotypes (Parker et al. 2014).

In a broad range of malignancies, several alterations in the network of microtubule have been detected, it includes varied countenance of tubulin isotypes, alteration in the post-translational modifications (PTMs) machineries of tubulins, and variation in the expression of microtubule-associated proteins (MAPs) (Kamal et al. 2020). Mutations in the tubulin are not generally common in the clinical conditions, though, the overwhelming data from in vitro research have shown the linkage between tubulin mutations and tolerance against tubulin-binding agents (TBAs). Although, in the growth and development of cancerous cells and its property of resistance against chemotherapy are not well understood; mutations in microtubules are hypothesized to impact cellular responses to chemotherapeutic stresses, therefore

imparting wide ambit of chemotherapy resistance, tumor growth, and cell survival (Parker et al. 2014).

Microtubule stabilizers, a member of PPI modulators (PPI stabilizers) are the compounds that modulate stabilization of microtubules and encourage their multimerization; they in turn, block the depolymerization of microtubules thus altering the tubulin dynamics. This further deteriorates the differentiation process in the mitotic phase, stalls the cell cycle, and triggers apoptosis in the tumor cells (Borys et al. 2020). Paclitaxel was the first microtubule stabilizer to be authorized which associates with α-tubulin and increases microtubule aggregation. It stabilizes microtubule structure and inhibits the spindle formation, causing cell cycle apprehension in G2/M phase (Alves et al. 2018). Similarly, the 20-membered macrolide, zampanolide is also reported to restrict the cells in mitosis and suppresses the cell growth by stabilizing microtubules (Roostalu and Surrey 2017). Apart from these, microtubule stabilizers derived from various natural sources prove to be effective in cancer therapy, greatly augmenting current generation-targeted medicines targeting the PPI between the isotypes of α-tubulin and β-tubulin. All indicators point to these medications continuing to be significant for treating cancer in the future (Karahalil et al. 2019).

2.19 Rac 1-GEF

Rac1 protein is a Rho GTPase that happens to be present in two states, GTP-bound and GDP-bound conformation, which are active and inactive forms of Rac1 protein respectively. Rac1 in its active state is significantly involved in the physiological processes such as cytoskeletal rearrangement, cell division, and migration of cell (Aznar and Lacal 2001). Guanine Nucleotide Exchange Factors (GEFs) and GTPase-Activating Proteins (GAPs) catalyze the switching of RAC-GTPase between active and inactive states (GTP-bound and GDP-bound). GEFs facilitate the interchange of GDP for GTP, resulting in conformational shift that displays the GTPase's effector-binding domain, allowing it to convey signals. Marei and coworkers have demonstrated that the selective suppression of the protein composite formed by Rac1 and its GEFs, including TRIO and TIAM1, would limit tumor invasiveness (Marei and Malliri 2017). Rac1 protein is highlighted as an important signaling hub that transduces the signal through the protein–protein interaction of Rac1 with EGF, platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) that are usually needed for many oncogenes to transform. Further, the recently discovered Rac1 gene mutant that facilitates the induction of skin cancer along with other types of malignancies also supports the fact that, it is one of the unappreciated drivers of cancer. Interestingly, in case of Rac1-GEF signaling cascade, mutant Rac1 protein is observed to be fast cycling than the conventional. Monitoring Rac1 and associated effectors can be an effective approach in this setting (Kazanietz and Caloca 2017).

Numerous drugs that disrupt GEF/RAC1 PPI have been investigated. NSC23766 and its analogs, such as EHop-016 and MBQ-167, are the best studied GEF/RAC1

PPI inhibitors. NSC23766 suppresses the machinery behind cell growth and transformation by hindering the functioning of TIAM1 and TRIO, restoring tumor cell phenotypes in prostate cancer cells while facilitating the unaffected CDC42 and RHOA activation (Zou et al. 2017). The most recent and promising molecule in this class is MBQ-167, which suppresses Rac1 activity while simultaneously inhibiting CDC42 activation. However, it is still indecisive whether MBQ-167 activity is totally associated with the activation of RAC pathways in tumor cells since the studies have been conducted on a very few cell types (Humphries-Bickley et al. 2017). Furthermore, other synthetic compounds developed to impair GEF/RAC1 complex are ZINC69391 and its derivative—1A-116 has been described to be involved in obstructing the communication of P–REX1 interface and interaction of GEFs with RAC1 and imparting antimetastatic benefits in breast cancer models (Ungefroren et al. 2018).

2.20 Sur2–ESX

The cell cycle progression and stimulation in all types of cancer are unabashedly related to the activity of growth factors and their regulation of expression. A classic example of this is HER2-positive breast cancer where overexpression of the HER2, a growth factor receptor has been linked with approximately 30% of the breast cancer patients and is resistant to conventional treatment (Menard et al. 2003). The regulation of HER2 expression and activity is tightly regulated through a transcription factor-coactivator system, ESX–Sur2. Hence regulation of ESX–Sur2 interaction is a smart approach for regulation of *her2* gene expression. ESX is a transcription factor which possess a conserved winged helix-turn-helix domain containing GGAA/T DNA-binding motif and a pointed (PNT) domain that interacts with Sur2, a Ras linked subunit of MED23 (human mediator complex). A short helical region of the ESX protein has previously been shown to impede the ESX–Sur 2 interactions in both in vitro and in vivo by Asada et al. (2002). In order to develop a small-molecule antagonist of HER2 expression, researchers adopted a semi-rational strategy that started with the predicted binding epitope. Adamanolol, a drug known to suppress ESX-dependent transcription, cell proliferation in HER2 expressing cells, and the production of HER2 itself, was shown to be effective at low micromolar doses inhibiting the Sur2-ESX PPI (Liu et al. 2011).

A second study used wrenchnolol (analog of adamanolol) to study the binding process, and it was discovered that the closed, s-*cis*-conformation is required for its inhibitory action. NMR-based analysis further indicates that, the hydrophobic ends of wrenchnolol are attached to Sur-2 as advocated by the design of the molecule (Dayam et al. 2007). Furthermore, the researchers employed a biotinylated analog in an experiment to detect the proteins in cell lysates exhibiting wrenchnolol-binding characteristics. Unsurprisingly, this chemical is modestly selective in cells, binding to a variety of proteins along with Sur-2. Adamanolol, and many other similar PPI inhibitors working against this protein-complex, is hydrophobic and massive (MW 800 Da). A further insightful study about specific selection of Sur-2 for

high-affinity inhibitors of the ESX–Sur-2 interface, that preferably possesses less biological consequences with higher selectivity is much appreciated (Yue et al. 2018).

2.21 CDK2/Cyclin A

Cyclin-dependent kinases (CDKs) are vital for cell cycle regulation mechanism, making them promising cancer therapeutic targets. CDKs are heterodimer protein kinase complexes made up of a kinase component that is catalytic in nature and a cyclin subunit exhibiting the regulatory functions. CDKs are divided into two classes based on their functions: cell cycle CDKs (e.g., CDK1, CDK2, CDK4, and CDK6), and transcriptional CDKs (e.g., CDK7, CDK8, and CDK9) (Wood and Endicott 2018). Members of the former group govern numerous cell cycle events in a sequential manner as shown in Fig. 2.10. In due course of process, active complexes are formed through protein–protein interaction between regulatory cyclins and catalytic CDKs. Among these PPIs, CDK2 complexed with cyclin A/E is pivotal in regulating the phenomenon of cell cycle. CDK2-cyclin A/E complex facilitates the process of DNA synthesis, evolution from G1 to S phases. Moreover, CDK2/cyclin A PPI is involved in the crucial cellular responses including DNA damage response and apoptosis (Asghar et al. 2015).

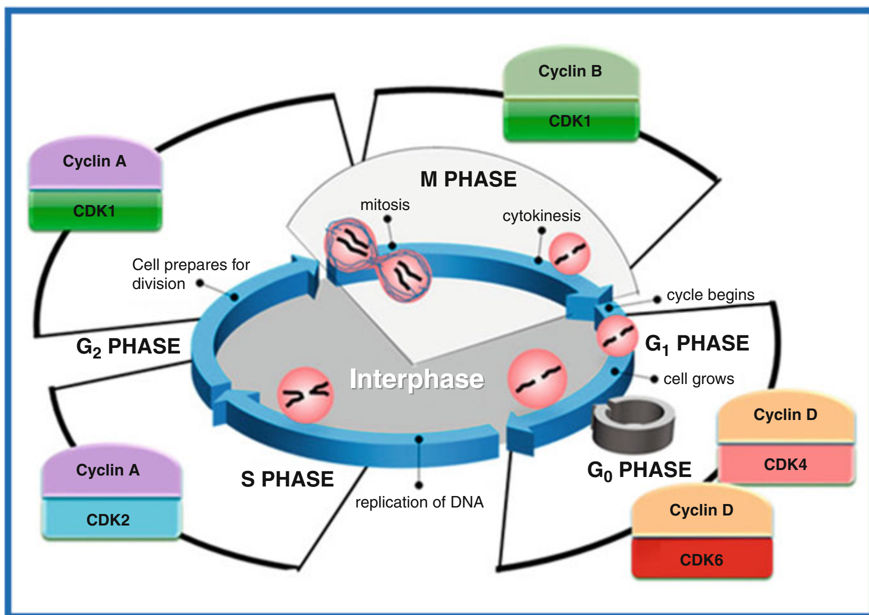

Fig. 2.10 Cell cycle phases and associated CDK/cyclin complexes. CDKs interact with specialized cyclin to develop an active complex that regulates the progression and translation of cell cycle into next phases. (Adapted from García-Reyes et al. 2018)

Subsequently, at the advance stage of S phase, several endogenous substrates are phosphorylated that assist DNA replication and interaction between E2F1 transcription factor and protein DP, which subsequently interacts with DNA thereby stimulating gene transcription. CDK2/cyclin A is necessary to phosphorylate E2F1 gene transcription, resulting in the liberation of the E2F1-DP complex and its eventual destruction (Peyressatre et al. 2015). CDK2/cyclin A inhibition keeps the E2F1 linked to DNA, resulting in chronic activation. As a consequence, the amount of E2F1 function will exceed the threshold value necessary to cause apoptosis independent of p53, thereby advocating a therapeutic approach. E2F1 is typically seen in high concentrations in cancer cells as a result of unregulated p53 and pRb pathways, therefore selective death in tumors might be a result of inhibition of CDK2/cyclin A, thus advocating this as a substantial target for anticancer molecules (Shapiro 2006).

Identifying the PPIs in the cyclin-binding groove (CBG) is a method for specifically inhibiting cell cycle over transcriptional CDKs. The CBG groove is a hydrophobic and located in cyclins A, D, and E that has been demonstrated to identify a consensus sequence present in substrates and tumor suppressors. The cyclin-binding sequence/motif (CBM) interacts with the CBG as an individual peptide and has been demonstrated to limit kinase activity of cell cycle CDKs (Premnath et al. 2016). Inhibiting the interacting interface of CBM/CBG PPIs would limit kinase activity of CDK2/cyclin A, which should cause cancer cells to undergo E2F1-mediated cell death while having no deleterious effect on normal cells. Taking breast cancer into the consideration, Ding et al. have reviewed the possible inhibitor and their mode of action that are targeting the CDK/Cyclin interaction for the treatment of cancer (Ding et al. 2020). Likewise, on a wide range, Chohan et al. have extensively discussed the probable protein–protein interaction in CDK/Cyclin-mediated cancer that can be used as the potential therapeutics for anticancer approach (Chohan et al. 2018).

2.22 HIF in Cancer

Hypoxia is a general attribute of several forms of solid tumors; due to the unregulated growth of the cells, the demands for the oxygen have outgrown the blood supply that would lead to the hypoxic conditions. In response, tumor cells activate a number of survival machineries that would help cancer cells to adapt the hypoxic environment. Hypoxia-inducible factor-1 (HIF-1) cascade is the best researched among them, its activation has been linked to stem cell viability, metabolic reprogramming, autocrine growth factor signaling, angiogenesis, metastasis, and also observed to impart the radiation and chemotherapy resistance (Fig. 2.11) (Li et al. 2019). Considering the structural aspects of HIF-1, it is a heterodimer of α-subunit (HIF-1α), and a β-subunit (HIF-1β). Both of these component proteins belong to the mammalian basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family of proteins (Yang et al. 2013). Under normoxia circumstances, HIF-1α is tightly controlled and eliminated by the ubiquitin proteome system comprising of

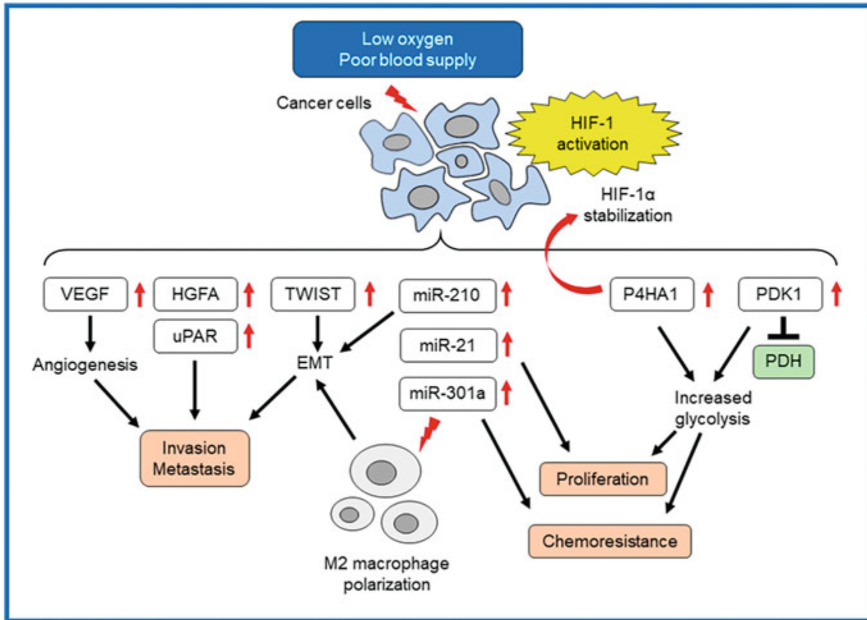

Fig. 2.11 Schematic showing the effect of hypoxia in cancer. HIF-1α interacts with numerous proteins and exerts the survival impact on the cancerous cells in hypoxic condition through invasion metastasis, increased proliferation, and chemoresistance. (Adapted from Hamada et al. 2022)

Prolyl-4-hydroxylase domain-containing enzymes (PHD1/2/3), as well as factor-inhibiting HIF-1 (FIH-1) to degrade HIF-1 (Stiehl et al. 2006). Contrary to this, under hypoxia, the activity of protease system is compromised that leads to the stabilization of HIF-α and its migration to nucleus where dimerization of HIF-α with its counterpart HIF-β takes place. The HIF-α/HIF-β dimer complex binds to the hypoxia response elements (HREs) on the target gene and promotes the recruitment of the coactivator (p300/CBP) to trigger the transactivation of target genes responsible for the adaptation of hypoxia conditions (Unwith et al. 2015).

In addition to this, the protein interactome in HIF system is also reported to be involved in the regulation of numerous hypoxia-induced proangiogenic mediators inside the tumor stroma and vascular endothelial cells (VECs), including vascular endothelial growth factor (VEGF), angiopoietin-2 (ANGPT2), stromal-derived factor 1a (SDF1a), stem cell factor (SCF), and platelet-derived growth factor-b (PDGF-b), which together play a critical role in initiation and progression of signaling cascade in tumor angiogenesis (Tiburcio et al. 2014). Another major interacting protein partner of HIF-1 is hexokinase 2 (HK2), which augments the glucose phosphorylation. Further, a group of glycolytic enzymes such as glyceraldehyde3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase (PGM), triosephosphate isomerase (TPI), phosphoglucose isomerase (PGI), phosphofructokinase 1 (PFK1), phosphoglycerate kinase (PGK), and pyruvate kinase (PK) are the

major interacting targets of HIF. Subsequently, HIF also forms a PPI network with lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4) that are involved in the reformation of lactate from the pyruvate and lactate elimination from the tumor cells (Yu et al. 2016).

Taken the aforementioned PPI network arbitrated through HIF system into account, numerous families of small molecules have been reported to hinder the PPI-like dimerization of HIF-1α/HIF-1β, HIF-1α/p300, and HIF-α/FIH, etc. Small-molecule acriflavine targets the dimer forming interface of HIF and destabilizes the heterodimer, this was one of very first anticancer drug to reach the phase II of the clinical trials (Lee et al. 2009). Further, a natural compound chetomin was reported to inhibit the interaction between HIF and p300 (Cook et al. 2009). On the similar note, various peptidomimetics of HIF that are having the micromolar affinity toward p300 have been proposed (Kyle et al. 2015). As discussed above, the HIF-1α is degraded in the normoxia condition through a regulated PPI between HIF-1α/pVHL; considering this, the approach of upregulating the protease activity in hypoxia condition has been explored. Through several structural-activity relationship-based studies, numerous PROTAC (Proteolysis targeting chimeric molecule) designated as compound 51 and compound 7 in studies have been discovered, and are in the different phase of clinical trials (Wilkins et al. 2016). A detailed mechanism of interaction between the aforementioned drugs and HIF has been explained in the Chaps. 6 and 7 of this book.

2.23 CBF in Cancer

Core-binding factors (CBFs) are the transcription factor complex proteins belonging to the class of hematopoietic transcription factors that play pivotal role in regulating hematopoietic ontogeny. The CBF complex is characterized as the heterodimer of two monomers, CBFA and CNFB designated as DNA-binding unit and non-DNA-binding unit respectively (Stengel et al. 2021). CBF binds with its interacting protein partner RUNX that belongs to the metazoan's transcription factor family and acts as the principal regulator of cellular development process. CBF-RUNX PPIs are obligatory in various developmental processes, such as proliferation, differentiation, apoptosis, and cell lineage determination. There are three RUNX genes in mammals: RUNX1, RUNX2, and RUNX3; each with different tissue-specific expression patterns (Ito 2008). RUNX genes are integrally implicated in carcinogenesis; RUNX1 mutations in human leukemia have been intensively researched, RUNX2 is a bone lineage-specific factor related to osteosarcoma, upsurge in the RUNX2 expression is also reported in the breast and prostate cancer cells, and RUNX3 in solid tumors is now well recognized as a tumor suppressors that are inactivated in the cancer cells (Ito et al. 2015).

Considering the critical role of CBF-RUNX complex in the cellular development and being the hotspot for the mutations that are critical for the cancer, CBF–RUNX interface can be the potential target for the small-molecule inhibitor of PPIs and other novel therapeutic approaches. In general, transcription factors are very difficult to

target, therefore, in the case of hampering the complexation of CBF–RUNX interactions, more emphasis was given to the inhibitors of RUNX factors. Pyrrole-imidazole polyamides, Ro5-3335 and AI-10-49 are some of the molecules that have shown promising antitumor effects in preclinical settings. These molecules precisely target the consensus RUNX motif in chromatin and prevent the interaction between CBF and RUNX (Otálora-Otálora et al. 2019).

2.24 FAK in Cancer

Focal adhesion kinase (FAK) is a protein tyrosine kinase that has been reported to predominantly control the integrin signaling pathways. Additionally, transmembrane receptors like G-protein-coupled receptor, cytokine receptor, and growth factor receptors, may work together to convey the cytosolic signals catalyzed by FAK. Structurally, FAK is organized into three major domains: The N-terminal band, the core kinase domain at the mid region and the focal adhesion targeting (FAT) domain situated at the C-terminal (Zhou et al. 2019). FAK regulates essential cellular developments such as adhesion, migration, proliferation, and survival of the cells through kinase-dependent and kinase-independent pathway (Fig. 2.12). In addition to this, FAK is also involved in the promotion of cancer stemness, tumor angiogenesis, and chemotherapeutic resistance (Murphy et al. 2019). Overexpression of FAK protein is demonstrated in ovarian, cervical, kidney, lung, pancreatic, brain, colon, breast, and skin cancers. FAK by its own is not the oncogene, but it is highly expressed in cancerous cells. There are pile of evidences establishing the fact that FAK PPI is allied to the initiation, survival, metastasis, and invasion of cancerous cells (Yoon et al. 2015).

Upon receiving the extracellular signal through transmembrane receptors (GPCRs, GFR, and CR), FAK is activated through self-phosphorylation of Y397 residue and transduces the subsequent signals responsible for various cellular functioning. In due course of process, FAK protein interacts with numerous other proteins and forms a protein–protein interactome that promotes oncogenic signals and inhibition of pathways responsible for tumor suppression. In light of this, FAK is considered as potential therapeutic target for the PPI inhibitors (Mousson et al. 2018). Several small molecules that are specifically targeting the FAK and allied proteins are under scrutiny. Among them, small molecules inhibiting the kinase domain of FAK are well characterized. These molecules bind competitively to the kinase domain of FAK and inhibit the binding of ATP, thus hampering the activation FAK downstream signaling cascades. Some of the well-studied examples of this category are GSK2256098, VS-4718, Defactinib, BI-853520, etc. Indeed, majority of these molecules are in Phase I and II of the clinical trials (Lu and Sun 2020). On the similar note, the FAK/p53 and FAK/ubiquitin E3 ligase H/MDM2 (human/mouse protein double minute 2) axis in the nucleus are the potential targets for the cancer treatment. In physiological condition, tumor suppression protein p53 interacts with the ubiquitin E3 ligase H/MDM2 (human/mouse protein double minute 2) which induces the proteasomal degradation of p53. Later it was reported that FAK

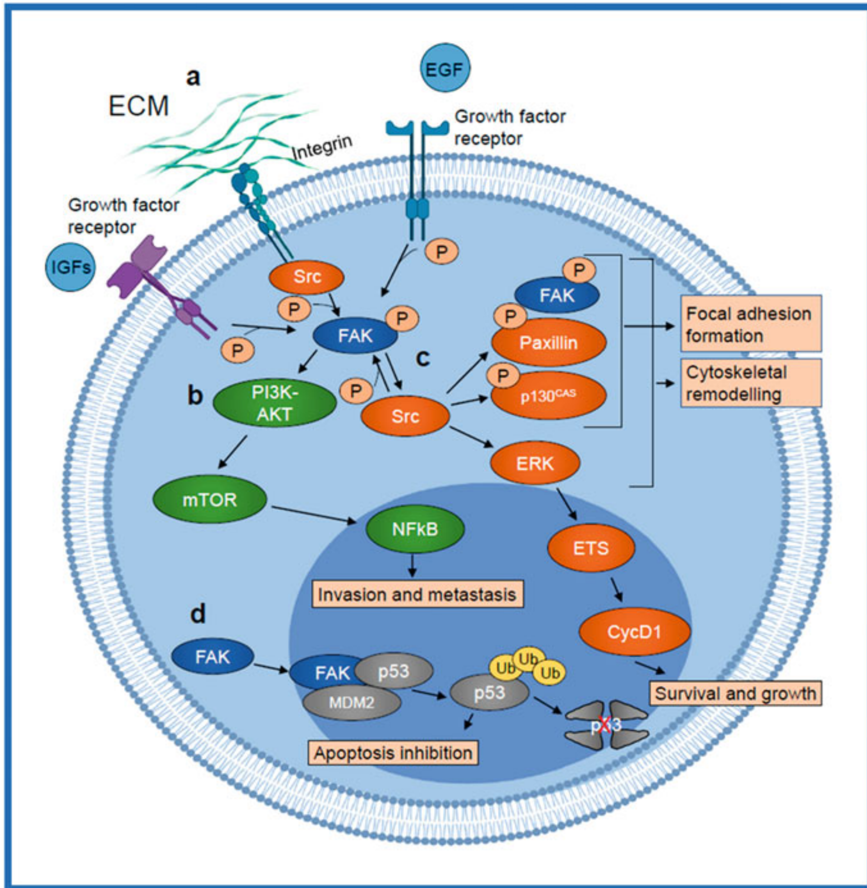

Fig. 2.12 FAK participation in tumor development and metastasis is shown diagrammatically. (a) FAK is auto phosphorylated as a consequence of the activation of growth factor receptors and integrins, and it is triggered by Src. (b) Active FAK stimulates tumor cell invasion and metastasis by activating the PI3K-AKT-mTOR signaling cascade, which increases NFκB transcriptional activity. (c) Active FAK also promotes cytoskeletal remodeling and focal adhesion formation/turnover by triggering Src-dependent phosphorylation of paxillin and p130cas, and results in the development of a focal adhesion complex including phosphorylated/active FAK, paxillin, and p130cas. Src also activates the ERK signaling cascade that leads to the ETS transcription factor-dependent activation of cyclin D1 (CycD1) expression, promoting tumor cell survival and proliferation. (d) Nuclear FAK functions as a scaffold protein for the p53–MDM2 interaction, promoting p53 ubiquitination and proteasomal degradation, which inhibits apoptosis. (Adapted from Perrone et al. 2020)

acts as the mediator for this PPI to facilitate the degradation of p53, thus regulating the cell survival (Pomella et al. 2022). Taken this into consideration, several small molecules hindering the complex formation between FAK-p53/MDM2 have been analyzed for their anticancer features. For example, Nutlin-3, AMG23, and MK-8242 bind to MDM2 with high affinity and inhibit the FAK/MDM2 complex

formation thus inhibiting the cancer initiation and development (Tisato et al. 2017). Along with the aforementioned PPI inhibitors, there are several other inhibitors under development that work in combination with therapies targeting allied signaling cascades and can be considered as an encouraging approach for the treatment of cancers.

2.25 B Cell Lymphoma 6 (BCL-6)

Humoral immune responses against foreign infections are majorly based on the germinal center (GC) response, which involves the development of high-affinity memory B cells and plasma cells. The formation of GC-B cells is extremely dynamic, guided by specialized and complicated transcriptional processes. The movement of B cells from the GC requires a series of gene expression, which are controlled by transcription factors that incorporate coactivator/corepressor conjugates to induce vigorous epigenetic and transcriptional alterations (Küppers 2005). Deregulating these molecular pathways disrupts the usual growth of B cells and contributes to the pathogenesis of majority of B-cell non-Hodgkin lymphomas (NHL), which include diffused large B cell lymphomas (DLBCLs), and follicular lymphoma (FL) (Huang and Melnick 2015).

The transcriptional repressor, BCL-6 acts as a master controller of the GC reaction, regulating its initiation and persistence (Hatzi and Melnick 2014). BCL-6 is obligatory for the growth and development of GC B-cells as well as T-follicular helper (Tfh) cells, a subtype of T helper that aids B cells during the GC response. BCL-6 protein is made up of three preserved provinces that are critical for its activity: BTB/POZ domain positioned at the N-terminal, which specifically interacts with corepressors such as BCOR; the central region having NCOR1, and NCOR2 comes into the contact with CTBP, NuRD, MTA2, and HDAC2; there do exist a zinc finger at the C-terminal domain, which interacts with specific DNA sequences. In consequence, these interactions allow BCL-6 to suppress transcription by recruiting transcriptional corepressor complexes, that in turn facilitate the repression of approximately 1000 target genes to occur (Huang et al. 2014). BCL-6 deficiency in any cell type causes the GC processes to terminate. BCL-6 operates as an oncogene in GC-derived B-cell lymphoma, wherein uncontrolled BCL-6 expression takes place owing to chromosomal relocation and/or mutations at the promoter sites, or by genetic abnormalities in BCL-6-regulated cascade (Yang and Green 2019).

There are different approaches of targeting BCL-6 for the treatment of cancers; (a) the use of compounds that interact with its C-terminal zinc-finger domain and restrict its binding with DNA; (b) downregulate the complete BCL-6 protein using RNAi, or small molecules that induce the proteolytic destruction. Though, researchers around the world have suggested that the complete destruction of BCL-6 would lead to the immunological complexities that are undesirable, and in worst condition might lead to death of the patients (Burotto et al. 2016). In a murine-based study, Toney et al. have described that in BCL-6 knockout mice, apart from downregulation of GC formation, there occur T-cell and macrophage-driven severe

systemic inflammatory diseases. In addition, it is also reported that BCL-6 degradation might cause the accelerated atherosclerosis and there occur huge permeation of inflammatory cells in lungs and added vital organs of the body that lead to death (Toney et al. 2000). However, it is feasible to prevent these deleterious effects via aiming at particular sites on BCL-6 that facilitates its cancer actions; nevertheless, keeping its anti-inflammatory capabilities unaffected.

At present, numerous PPI inhibitors have been demonstrated that directly interact with the BCL-6 and hinder its interactions with other counterparts, thus hampering the proliferation of GC B cells. For example, compound GSK137 is proved to interact with the BTB domain of the BCL-6 with high affinity and block the corepressor binding (Pearce et al. 2021). Similarly, Kamada et al. used biophysics-driven fragment-based drug discovery approach (FBDD) for generating the pyrimidine derivatives that targets different PPIs in BCL-6-mediated GC response (Kamada et al. 2017). Likewise, many compounds such as 79-6, WK500B, and FX1 show very high affinity toward the BTB domain of BCL-6, and proved to be a potential drug leads for the treatment of B-cell non-Hodgkin lymphomas (Xing et al. 2022).

2.26 Notch Receptors

Notch receptors-mediated signaling pathways are the part of a highly conserved system in multicellular organisms that depend on cell–cell interactions to trigger a response. During development and homeostasis, Notch signaling controls the cellular fate of the tissues and directs it either to the lineage commitment, differentiation, cell cycle progression, stem cell perpetuation, and self-resumption (Nowell and Radtke 2017). In mammals, there exist four Notch receptors (Notch 1–4) and five ligands including jagged 1 (JAG1), JAG2, delta-like 1 (DLL1), DLL3, and DLL4. The structural details of the Notch receptors suggest that these are the integral membrane heterodimers, having a ligand-specific extracellular domain and an intracellular region that triggers the signaling upon binding of ligands (Capaccione and Pine 2013). In Notch-dependent signaling cascades, the phenomenon of interaction between ligand and receptor can be classified as *cis* and *trans*; in the latter case, the ligands and receptor are present on the nearby cells, while in the former both are present on the same cells. The *trans* interaction leads to the activation of canonical pathway (Fig. 2.13), results in the activation of metalloproteinase that dissolutes the extracellular ligand-specific domain of the receptor, keeping the intracellular part bound to the plasma membrane. Over the time, intracellular domain of Notch (NICD) receptor is also disintegrated from the transmembrane domain by the activity of secretase composite and free NICD translocates to the nucleus and connects with the DNA-binding proteins to form a Notch transcriptional complex (NTC). Further, coactivators like mastermind-like protein (MAML) and p300 are attracted toward the NTC and thereby facilitate the transcription of genes (HES1 and HEY1) dictating the commitment of cell lineage (Yuan et al. 2015). Apart from the canonical pathway for Notch signaling activation, researchers have identified several unrelated proteins

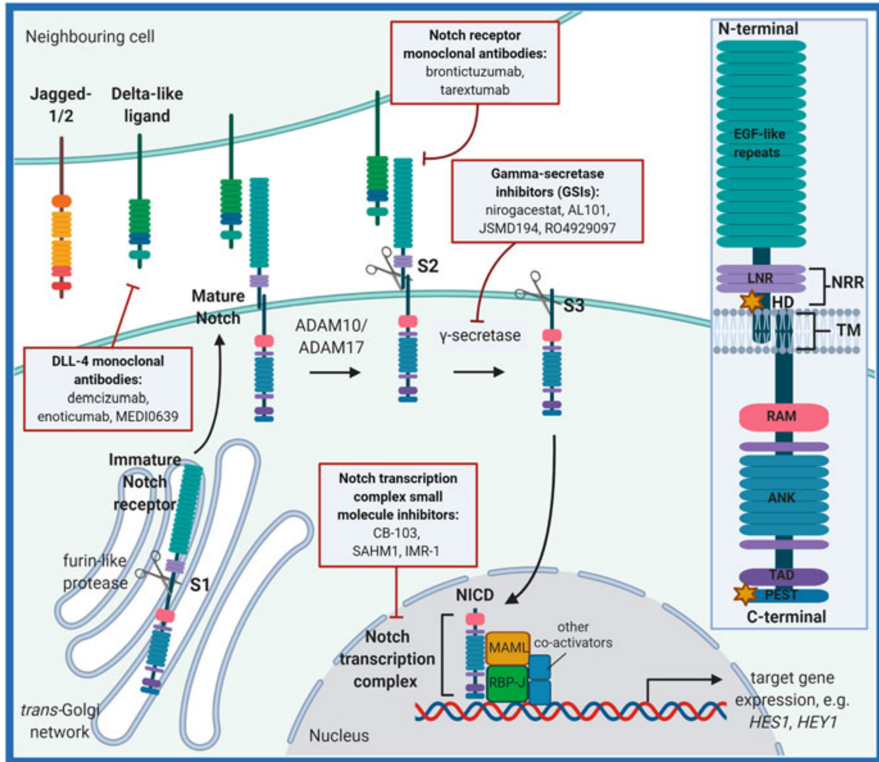

Fig. 2.13 Notch signaling and pharmaceutical techniques for directing this route. The mature heterodimeric Notch receptor is maintained by NRR after a furin-like protease cleaves the immature notch receptor at site S1 inside the HD domain, positioned amid the LNR and the TM. Notch-specific ligands bind to its cognate receptor at the regions of N-terminal, lead to its activation. The intracellular part denoted as NICD is freed and migrates to the nuclear region, where it specifically binds with RBP-J, MAML, and other coactivator proteins and enzymes, that in turn encourage the transcription of notch target genes such as the hairy/enhance of split 1 (HES) and Hes-related (HEY) families of transcription repressors. (Adapted from Moore et al. 2020)

(NB3/Contactin6 and MAGP1/2) to establish an interactome with Notch receptor and allied proteins for activating the signaling cascade (Bazzoni and Bentivegna 2019).

Given the significance of Notch signaling in the functional regulation of stem and progenitor cells, it is perchance predictable that the dysregulation in Notch pathway through germline and somatic mutation would lead to the development of various cancers. Aberrant Notch signaling leads to the unregulated proliferation and differentiation of hematopoietic stem cells (HSCs) that would lead to the carcinogenesis. In line with this, T cell acute lymphoblastic leukemia (T-ALL), where Notch operates as a true oncogene, is perhaps the most studied cancer induced by Notch signaling. Hotspot mutations in the ligand-binding regions of Notch receptors were first discovered in T-ALL (Sanchez-Martin et al. 2017). Aster et al. reviewed a broad

variety of mutations and gene modifications that impact the PPI of Notch with its interacting protein partners, resulting in Notch receptor hyperactivation, indicating the broad therapeutic potential of Notch (Aster et al. 2017). Notch being an important aspect of cell-to-cell interactions amid cancer cells as well as cells in the tumor niche, it exhibits a cross talk with various added oncogenic signaling pathways and may provide acquired resistance to therapeutic strategies and also aversion to standard chemo/radiotherapy. It could be pharmacologically targeted using a variety of approaches, such as monoclonal antibodies developed against Notch receptor, antibodies definite to ligand (e.g., DLL-4 antibodies), gamma secretase inhibitors (GSIs), and inhibitors of Notch transcription complex (Moore et al. 2020).

Till date, several PPI inhibitors targeting the different axis of the notch signaling pathways have been developed. Secretase (α/γ) inhibitors are the class of inhibitor that targets the interaction between the intracellular region of Notch and secretase complex, thus hinders the release of free NICD from the receptor. DAPT (GSI-IX) is one of the secretase inhibitors that is reported to synergistically amplify the effect of radiation treatment and also suppresses the proliferation of cells (Bazzoni and Bentivegna 2019). Similarly, arsenic trioxide (ATO), Tipifarnib, Honokiol, Fibulin-3, and Protein kinase C iota are some of the anticancer molecules reported to inhibit the PPI interaction at different stages of Notch signaling cascade. Over the last two decades, more than 70 anticancer clinical studies have been filed, and nirogacestat is the first-ever to reach the Phase III trials (Moore et al. 2020).

2.27 Conclusion

Cancer is a very diverse illness that causes multilayer cellular transformations that encourage uncontrolled cell proliferation. Individual cancer cell's genetic and epigenetic modifications show up as variations in protein–protein interactions (PPIs), which reconfigure many of the signaling and regulatory pathways in cells. It is noteworthy that these protein complexes are essential for each phase of the fundamental aspects of genetic transmission, the key tenet of molecular biology. To further understand and confirm the druggability of protein complexes relevant to the core dogma, novel chemical probes must be identified in light of the numerous significant discoveries being made about the mechanisms underlying these genetic processes. In this chapter, an overview of how cancer develops and spreads, as well as the importance of the main proteins and PPIs involved in the procedure has been discussed in detail. It is worth noting that the potential targets for the small molecules used in the treatment of cancer majorly include these protein complexes and their corresponding partners.

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3 Protein–Protein Interactions in Neurodegenerative Diseases

3.1 Fundamentals of Neurodegeneration

Neurodegeneration is the gradual loss of structural and/or functional aspects of neuron, leading to cell death which may eventually result in a variety of clinical and pathological manifestations, as well as degradation of functional anatomy (Przedborski et al. 2003). The progressive neuronal cell death frequently results in neurodegenerative disorders (NDDs) that includes Parkinson's (PD), Huntington's (HD), Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), spinocerebellar ataxia (SCA), Spinal Muscular Atrophy (SMA), and others, which can be distinguished on the basis of the cognate arbitrary pathological pathways (Poddar et al. 2021). Traditionally, each of these illnesses were considered as separate entity with well-defined clinical and pathological characteristics. Neurodegeneration is well-known for causing memory loss, depriving individuals from their quality of life and fundamental individuality. Although, it can be distinguished by the miscommunication between brain cells, due to its complexity, the cause of these diseases is still unknown. Almost all the neurodegenerative diseases are predominantly distinguished by the presence of filamentous inclusions. Each type of inclusion comprises of at least one of three neuronal proteins—amyloid-β, tau, and α-synuclein, as its major component (Jucker and Walker 2018). The term "neurodegenerative disease" implies a class of disorders that predominantly damage the neurons in the human brain. It is an all-too-common collection of symptoms that individuals experience as they become older. NDDs are defined by the gradual malfunctioning of synaptic nerves, neurons, glial cells, and complex network formed by them. The deposition of physicochemically altered forms of proteins involved in the physiological aspects of nervous system is a critical component of NDDs. Along with neurons, glial cells also, acquire these pathogenic proteins (Kovacs 2016). The variable clinical symptoms of NDDs, along with the discovered molecular pathological origins of disease-associated proteins, necessitate studying the interaction of these proteins

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with their companion proteins for appropriate diagnosis and treatment (Dugger et al. 2019).

Millions of elderly individuals across the globe suffer from these neurodegenerative illnesses, with the diagnosis and treatment of these diseases costing billions of dollars each year in medical expenses (Kovacs 2016; Hammond et al. 2019). According to a recent WHO study, the overall number of NDD cases worldwide are expected to be about 50 million, with 60% of cases occurring in economically weaker countries, and ten million new cases being recorded per year. NDDs are classified based on their clinical manifestations, the anatomical areas, type of the cells that are more influenced, the conformationally changed proteins participating in the pathogenic processes, and, if known, the etiology (i.e., genetic differences) (Kovacs 2018). There are two important points to remember: (1) The anatomical location demonstrating neuronal malfunction that determines the clinical symptoms, and not necessarily the dispersal of the altered protein; and (2) the proteins linked to NDD undergo a broad range of biochemical changes and may aggregate in the intracellular region of neuronal and glial cells or accumulate in extracellular place like plaques, counting those that demonstrate amylogenesis. Identification of the anatomical, cellular, and protein susceptibility patterns in NDDs appears to be the most efficacious approaches till date (Armstrong et al. 2005; Kovacs and Kovacs 2015).

Clinical sign of neurodegeneration may begin either with dementia, intellectual loss, and abnormalities in higher-order brain processes (such as signaling in neocortical regions and limbic system) or with locomotion disorders distinguished by dysfunction of motor neurons. In most of the cases, the combination of both are observed in the early stage (Kovacs et al. 2014). Molecular pathological categorization of the neurodegenerative disorders is based on the difference in the synaptic nerve and intra/extracellular accumulation of the proteins (Kovacs et al. 2010). These proteins include but are not limited to (1) amyloid-beta (Aβ), formed through dissociation of APP (amyloid precursor protein), a 110 kDa protein mapped on the chromosome 21q21.3. (2) *snca* gene-encoded α-synuclein protein on chromosome 4. (3) *prnp* gene-encoded prion protein (PrP) positioned on chromosome 20. (4) Tau protein linked to microtubules, encoded by *mapt* gene situated on chromosome 17q21 (Kovacs 2019) as shown in Table 3.1.

NDD is a wide spectrum of disorders where the pathophysiology and symptoms overlap, particularly in multisystem atrophy, when multiple regions are damaged at the same time, making clinical analysis challenging. NDDs are diagnosed by the thorough observation of anatomical and biochemical changes that correlate with neuronal dysfunction, alterations in biomarkers (both protein and DNA), protein deposition, and genetic and epigenetic modifications (Kovacs and Kovacs 2015). In addition to prevention, several compounds, such as antibodies, chaperones, nanoparticles such as tetracyclines nanoparticles, and metal chelators, have been used for the treatment of NDDs by altering the aggregation processes of diverse amyloidogenic proteins, like Aβ, α-synuclein, PrP protein, etc. (Arosio et al. 2014). However, the most difficult aspects of therapeutic strategies where research emphasis is required are: (a) preventing oligomer formation or adjusting the aggregation

Table 3.1 Summary of the proteins involved in the major neurodegenerative disorders

Sl No.	Protein	Location of protein	Disease	References
1.1.	Tau	Neurons	Alzheimer's disease (AD) Pick's disease Dementia NBIA (Neurodegeneration with brain ion accumulation)	Gao et al. (2018)
		Glia	Globular glial tauopathies	
2.	Amyloid-β (Aβ)	Extracellular	Alzheimer's disease	Gupta and Goyal (2016)
1.3.	α-Synuclein	Neuron	Parkinson disease (PD)	Bennett (2005)
		Glia	Multiple system atrophy	
4.	Huntingtin	Neurons	Huntington's disease (HD)	Bano et al. (2011)
5.	PrP	Extracellular and synaptic	Prions disease	Jaunmuktane and Brandner (2020)
6.	Ataxins	Neurons	Spinocerebellar ataxia (SCA)	Bolger et al. (2007)
7.	TDP-43	Neurons and glia	Motor neuron disorders	Jo et al. (2020)

into nontoxic cascade, (b) regulating the migration of therapeutic compounds across blood–brain barrier (BBB), and (c) site-specific translocation of drug-loaded nanoparticles at the specific neuronal site to lessen side effects and toxicity that are dose-dependent. The advancements in biochemical, pathological, and pharmacological research may guarantee a brighter future for global neuronal health, nonetheless it requires the adoption of a healthy lifestyle from infancy to prevent the development of such NDDs as the concern of neurodegenerative disorders is both social and scientific in nature.

3.2 Interacting Proteome of Synapse

The word synapse refers to the connection between neurons and its target effector cells in the nervous system that conveys information encoded with action potential. They are primarily classified as electrical, and chemical synapses based upon the type of transmitter used. Chemical synapses consist of a synaptic cleft, present in between the pre- and postsynaptic terminals which are highly specialized structures for rapid and accurate unidirectional signal transduction. While, in the case of electrical synapse, there is direct transmission of charged ions and tiny molecules through pores called gap junctions (O'Rourke et al. 2012). Though having distinct morphology, roles, and compositions, the synapses are organized around a presynaptic terminal loaded with vesicles containing neurotransmitters that is precisely juxtaposed to postsynaptic compartment equipped with varied range of receptors on its surface that transduce the signals received from presynaptic terminals thus activating the numerous cellular processes. These two regions are joined together

through synaptic cell adhesion molecules lying in the region of synaptic cleft (Sheng and Kim 2011; Pinto and Almeida 2016).

Being the smallest unit in the CNS, synapse interrelates numerous neurons in the circuit and helps in the progression of neuronal communication and healthy functioning of the brain. Synaptic anomalies have also been linked in growing number of neurodegenerative disorders along with the developmental disorders (Taoufik et al. 2018). Synaptic dysfunctions occur mainly due to the alterations in the intrinsic molecular mechanism and variation in characteristic biochemical phenomenon of the cells. The diseases in which synaptic dysfunctions are crucially involved, collectively termed as synaptopathies. This is the mutually used term for the neurodegenerative diseases like AD, PD, and prion pathologies (Lepeta et al. 2016; Ardiles et al. 2017). Regardless of having very smaller size, synapse is remarkably complex to deal with, it involves hundreds of proteins, whose spatiotemporal expression and dynamics remain an unnerving challenge for scholars. A proteomics study of synapse scrutinizes the molecular mechanism involved in the neurodegeneration and is applied to search of biomarkers for various NDDs (Xu et al. 2021).

Though mapping the proteome is an essential first step toward an inclusive knowledge of synapses, comprehending synaptic signaling requires study of the structure and governing responsibility of synaptic proteins in a purposeful context. Due to the intrinsic benefits of MS-based proteomic methods for investigating PPIs and PTMs, they have been already become an essential apparatus for analyzing protein complexes and protein dynamics during synaptic signal transmission (Lepeta et al. 2016; Xu et al. 2021). Multiprotein complexes are critical for the synapse's structure and function. They provide distinct intracellular microenvironments conducive to protein interaction and reaction precision and efficiency. The postsynaptic density (PSD) is a dynamically specialized complex instituted in the postsynaptic terminal region and till now, more than 1000 proteins have been studied in this region (Torres et al. 2017).

Presynaptic components found in the distal terminal of the axon connect the point of release for neurotransmitters. The presynaptic terminal's activity is tightly controlled and has been linked to brain function and NDDs such as AD and PD (Bridi and Hirth 2018; Ivanov et al. 2018; Barthet and Mulle 2020). The molecular atlas of this neuronal sub-compartment obtained using combined proteomics and systems biology techniques, leads to a principal list of 117 existing and 92 anticipated presynaptic proteins (Abul-Husn et al. 2009). Proteomics has also been used to identify proteins expressed in presynaptical mitochondria, crucially involved in the development of brain. Forty mitochondrial proteins were found to be variably expressed in mice during postnatal days 7–42. Cisd1 (MitoNEET) was one among them that was proved to be the major regulator of mitochondrial activity (Stauch et al. 2019). Under some pathological circumstances, the presynaptic proteome becomes unregulated. For instance, in a hippocampal synaptic proteome mice model, it was well explained that the expression of presynaptic markers decreased in cognitive defects, confirming the altered presynaptic architecture and aberrant synaptic function in this brain region (Lenselink et al. 2015). Similar to this,

Table 3.2 Summary of the proteins involved in the major neurodegenerative disorders

Protein	Function	Associated neurological disorders	References
Presynaptic			
Synpasin-I, Synpasin-II/ Synpasin-III	Chemical synaptic transmission and regulation, neurotransmitter secretion	AD, HD, ALS, PD	Mirza and Zahid (2018), Bridi and Hirth (2018)
Synaptotagmin-1	Maintaining the intact synaptic transmission	AD, PD	Öhrfelt et al. (2016), Bridi and Hirth (2018)
Synaptophysin	Regulating the neurotransmitter release and synaptic vesicles endocytosis	AD, PD	Bridi and Hirth (2018), Russell et al. (2012)
Rab3-interacting molecules (RIMS)	Synaptic vesicle fusion, and neurotransmitter release	PD	Bridi and Hirth (2018)
SNARE proteins	Synaptic vesicle fusion	AD, PD	Margiotta (2021)
Cadherin	Synapse formation	AD, PD	Seong et al. (2015)
Postsynaptic			
Adhesion molecule (NL1/2/3/4)	Formation, maturation, and remodeling of inhibitory synapse	AD	Tristán-Clavijo et al. (2015)
Glutamate receptors (NMDARs, KARs, AMPARs, mGluRs)	Regulating the plasticity of synapse, maturation of neural circuit	AD, HD	Mota et al. (2014), Milnerwood et al. (2010), Mandal et al. (2011)
Scaffolding proteins (shank1/2/ 3, PSD-95)	Stabilization of the synapse, regulating the molecular structure, and modulation of interacting proteins in the PSD	AD, HD, PD	Bridi and Hirth (2018), Leuba et al. (2014), Murmu et al. (2015)

AD, Alzheimer's diseases; *HD,* Huntington's diseases; *ALS,* amylotrophic lateral sclerosis; *PD,* Parkinson's diseases

numerous additional presynaptic proteins (not limited to) mentioned in Table 3.2 are also involved in NDDs.

The gap between pre- and postsynaptic terminals, known as the synaptic cleft, it is an essential section of the synapse in terms of its structure and functions. Though, the difficulty to biochemically separate the clefts makes characterization difficult, new proximity-labeling proteomics methods are allowing researchers to investigate the protein component and alterations in this specialized section (Xu et al. 2021). Researchers discovered that the synaptic cleft contains many ion channels, GPCRs, adhesion proteins, transporters, as well as novel cleft contestants. Cijsouw and coworkers have explored the distinctions between excitatory glutamatergic and inhibitory GABAergic synaptic clefts using peroxidase-mediated biotin-labeling methods (Cijsouw et al. 2018). These results not only confirmed the utility of vicinity-labeling methods designed for deciphering the molecular features of

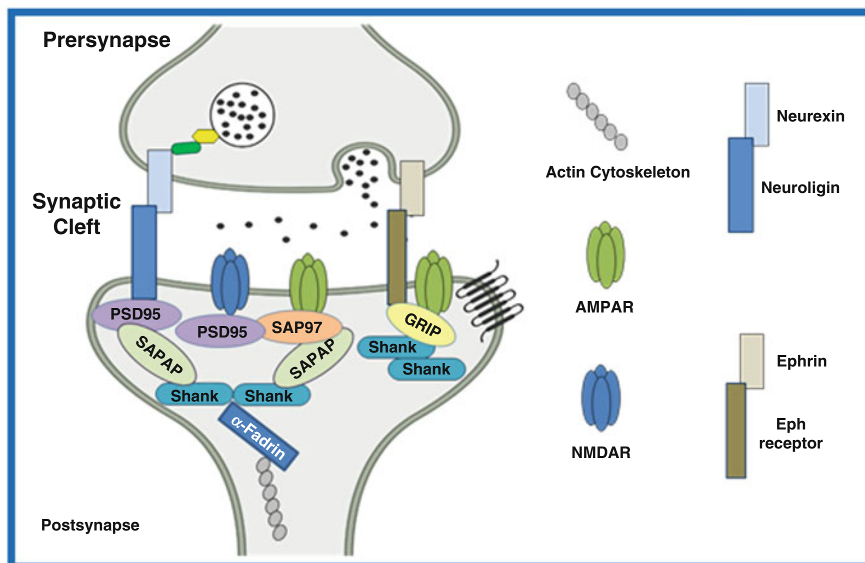

Fig. 3.1 Major proteins in the postsynaptic density (PSD) region. Postsynaptic membrane has receptor-like AMPA (alpha-amino-3-hydroxy-5methyl-4-isoxazole-propionate) and NMDA (*N*-methyl-D-aspartate) that are involved in the detection of glutamate that is synaptically released. Proteins like PSD95, SAP97, and GRIP are the membrane-associated kinases that are involved in the regulation of this surface receptor. Shank protein is responsible for the generation of multimeric sheet that maintains the presynaptic structure and function

previously unpurifiable structures, but also added new information about the synaptic communication igniting new biological ideas such as the putative contribution of Mdga2 as a synaptic inhibitory specificity factor (Loh et al. 2016).

On the postsynaptic side, there is a complex known as postsynaptic density (PSD), which is a microscopic structure connected with the membrane (Palay 1956). Over 2100 proteins have been found and measured in the last several decades, providing a complete picture of this protein dominant compartment, some of the significant proteins are shown in Fig. 3.1 (Distler et al. 2014; Wilson et al. 2019). As PSD scaffolding proteins are majorly involved in glutamate receptor trafficking and scaffolding, they may be involved in synaptic pathology of neurological developmental and degenerative illnesses. In rodent models of various NDDs (AD, PD, HD, ALS), SAP97, PSD93, PSD95, SAP102, and Shanks subcellular localization and expression levels are dramatically changed (Fourie et al. 2014; Vyas and Montgomery 2016). In these NDDs, abnormal protein–protein interaction like SAP97–ADAM10 connections is also disturbed to promote amyloid protein plaque development (Musardo et al. 2014).

Along with the NDDs, PSD proteins are now being recognized as important contributors to neurodevelopmental diseases. Multiple neurodevelopmental problems such as autism spectrum disorders (ASD), Phelan-McDermid syndrome,

and schizophrenia, have been linked to mutations in the Shank family of proteins (Vyas and Montgomery 2016). While PSD proteins are obviously altered by the illness process, their diverse functional aspects are because of splice variants, and expression patterns provide promising possibilities for brain healing. Due to the failure of pharmacological targeting of glutamate receptors in the treatment of neurological illnesses, new research has focused on PSD proteins to validate their utility as therapeutic targets to change glutamatergic synapses. Modification of PSD protein interactions with their binding partners using tiny cell permeable peptides has shown to be a promising therapeutic method with little adverse effects (Zhou et al. 2015).

3.3 Alzheimer's Disease (AD)

The deterioration of memory, intelligence, and cognitive ability, along with personality and behavioral changes, are the common indicators of dementia clinically termed as Alzheimer's disease (Ganeshpurkar et al. 2019; Heinemann et al. 2010). AD causes gradual neurodegeneration characterized by nerve cell injury which directs to uncanny neuropathological and neurochemical circumstances (Bruggink et al. 2012). This is a frequently occurring type of dementia majorly observed in elderly people. AD is broadly classified into two types based on the cause of the disease: early-onset familial Alzheimer's disease (eFAD) and nonfamilial/late-onset Alzheimer's disease (LOAD). Even though eFAD and LOAD appear to be identical on the surface, the causal process that leads to eFAD is not the same which leads to LOAD. As a hereditary ailment, eFAD is unambiguously the result of malfunctioning in mutant genes, while LOAD is more likely the result of a gradual accumulation of age-related breakdowns (Bird 2012; Hardy 2006; Rhinn et al. 2013). A study on AD established that, in addition to age and inheritance, lifestyle is also a major contributor in the advancement of the disease (van Praag 2018).

As Alzheimer's disease progresses, the brain cells (both neuronal and nonneuronal) get affected, and various parts of the brain are observed to shrink. The damage usually originates in the memory-controlling area of the brain, although the process can begin years before the first symptoms appear. The loss of neuron spreads to other areas of the brain in a rather predictable fashion. The brain shrinks greatly at the late stage of the illness (Realdon et al. 2016). Intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques are the conventional attributes of this disease. These proteins' deposits are made up of hyperphosphorylated microtubule-linked protein tau (p-τ) and amyloid-β (Aβ) peptide aggregates as shown in the Fig. 3.2 (Kovacs et al. 2014). A variety of mechanisms entail the generation of Aβ and the phosphorylation of the tau protein. The buildup of Aβ is caused by the caspases cleaving the amyloid precursor proteins (APPs) in tandem, whereas tau hyperphosphorylation is because of the actions performed by the multiple kinases. Indeed, the mounting data suggest that these aberrant protein deposits interact with one another and have a cumulative impact on the development and progression of AD (O'Brien and Wong 2011). It is also evident

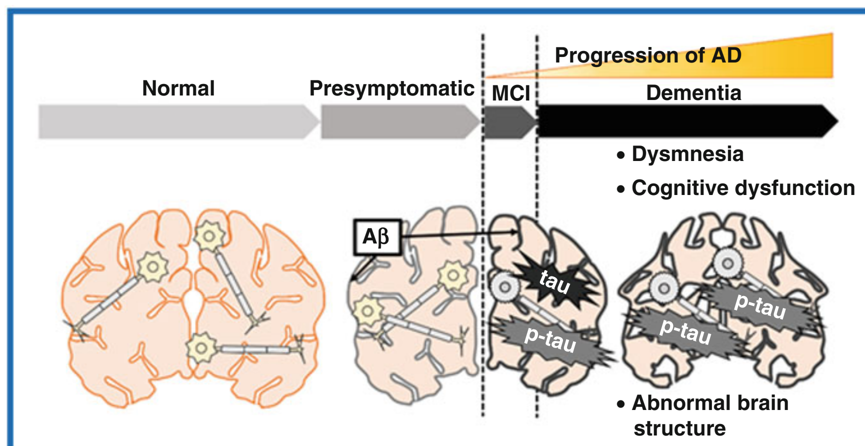

Fig. 3.2 Etiology of Alzheimer's disease; AD is categorized by the deposition of tau, p-tau, and amyloid-β (Aβ). (Adapted from Gunes et al. 2022)

in most cases that Aβ deposition controls the pathogenesis of tau protein. The advancement in biological sciences, along with computational approaches, have paved the path for studying the different proteins and PPIs involved in the development of AD (Ganeshpurkar et al. 2019). The pathophysiological role of these proteins, their interaction, and the outcome of these PPIs are discussed below. Most of these could be used as encouraging targets for drug development against AD.

3.3.1 Cyclin-Dependent Kinase 5

There are numbers of kinase-mediated metabolic pathways involved in Alzheimer's disease. These pathways include AMPK (5′ Adenosine Monophosphate-activated kinase), Fyn kinase (Yang et al. 2011), Wnt signaling, and cyclin-dependent kinase 5 (Cdk5) signaling. Wnt signaling in general possesses the neuroprotective traits, but as the disease progresses, various components (β-catenin, Dickkoff-1, apo-lipoprotein E) of the Wnt signaling are affected that leads to the worsening of the condition (De Ferrari and Inestrosa 2000). AMPK is a serine/threonine kinase that primarily preserves cellular energy levels by metabolizing glucose and lipids. Fascinatingly, neuronal energy metabolism aberration such as drop in glucose uptake, defects in cholesterol metabolism, and malfunctioning of mitochondria are crucially observed in AD. This phosphorylates the tau protein, and results in neurofibrillary tangles (Zhu et al. 2002).

Amongst the aforementioned kinases, Cdk5 has a prominent role in the pathological advancement of AD. Cdk5, is a proline-directed Ser/Thr kinase, functionally responsible for the neuronal plasticity, neuron development, and survival (Mushtaq et al. 2016). Cdk5 is abundantly found in postmitotic neurons, where it is specifically

activated through neuronal membrane localized activators—p35 and p39. The Cdk5-activators are highly prone to ubiquitin-mediated proteasomal degradation and have a short half-life. Both p35 and p39 comprise of two polypeptide regions: p10 and p25/p29 respectively generated after cleavage by calpains under high calcium concentration (Liu et al. 2016). The p35 protein is the most well-studied interactor of Cdk5 which interacts in both p35 and p25 forms. Chemical modifications such as phosphorylation and S-nitrosylation also play an important role in defining the Cdk5 kinase activity. Three residues namely Thr14, Tyr15, and Ser159 are major sites for phosphorylation and S-nitrosylation. The modification on these three sites either heightens or inhibits the Cdk5 activity (Ramesh et al. 2020). Cdk5 relays its activity via phosphorylation of the consensus sequences namely KSPXX, and KSPXK found in neurofilaments, and microtubule-related proteins. Further Cdk5 is an autoinhibitory protein which is involved in a feedback loop, where it phosphorylates the p35 protein at Ser8 and Thr138 position (Shah and Lahiri 2014).

Cdk5 is essential in both neuronal survival and death, the duality of function is dependent on presence of either p35 or p25 form. The CDK5/p35 performs neuroprotective functions while CDK5/p25 is involved in cell death and neurodegeneration process (Pao and Tsai 2021). The presence of p35/p25 is regulated by the cytoplasmic calcium levels. Pathological/stress conditions result in increased level of calcium in neurons, which activates the calpains for dissociation of p10 and p25. In p25 form, it establishes an increasingly stable and hyperactive complex with CDK5 called the CDK5/p25 complex. This complex facilitates its localization in cell soma and nucleus, and causes hyperphosphorylation of tau protein resulting in cell death and neurodegeneration (Lee et al. 2000). The Cdk5/p25 complex initiates numerous cell death pathways that result in oxidative stress, mitochondrial dysfunction, and elevates the calcium influx. For instance, the Cdk5/p25 complex activates and phosphorylates peroxiredoxin-1, peroxiredoxin-2, and NMDAR (*N*-methyl-D-aspartate receptors) responsible for neuronal cytotoxicity and cell death (Malhotra et al. 2021; Shukla et al. 2017). Moreover, Cdk5 inactivates myocyte enhancer factor 2 (MEF2) by phosphorylation of Ser444 which makes cortical neurons susceptible toward oxidative stress (Gong et al. 2003). Cdk5 is also predominantly involved in the regulation of DNA damage and DNA repair mechanisms. Acetylation of Cdk5 at Lys33, phosphorylation of Ape1, and dopamine cyclic AMP-regulated phosphoprotein (DARPP-32) which is a PKA inhibitor greatly influence the DNA repair process and lead to DNA damage and neuronal cell death (Lee et al. 2014).

One of the main components of AD pathology is the buildup of amyloid-β peptide (senile plaque). Aβ is a result of successive cleavage of integral membrane glycoprotein, i.e., APP by β-secretase (BACE1) followed by γ-secretase, which liberates Aβ peptides. Cdk5 regulates APP synthesis and plaques form as the extracellular deposits of Aβ peptides accumulate (Nhan et al. 2015). Other pathogenic features of AD include neurofibrillary tangles which are hyperphosphorylated cytoskeleton of tau proteins. Cdk5 kinase activity in tau phosphorylation is considerably greater in the presence of p25 in comparison of p35, which exhibits the lessened axonal transports (Zhou et al. 2010; Kimura et al. 2014). Cdk5/p25 complex phosphorylates

numerous epitopes of tau, including Ser202, Thr205, Ser235, and Ser404, which are potential hyperphosphorylation sites in Alzheimer's disease (Kimura et al. 2014). Other than these main signaling cascades, Cdk5 indirectly regulates numerous pathways responsible for the advancement of AD. Because of its role in tau pathology, tangle, and plaque formation, Cdk5 is a potent target for therapeutic agents against AD.

3.3.2 Calpains

Calpain proteases belong to the cytoplasmic nonlysosomal calcium-dependent cysteine protease family that regulates structure of the cytoskeleton and intracellular signaling pathways in the cell (Goll et al. 2003). They are regulated by Ca^{2+} and calpastatin (CAST), which activate and inhibit them, respectively (Ono and Sorimachi 2012). Stability of this regulatory mechanism appears to be reduced with age, causing unregulated calpain activation, implying the role of these enzymes in various illness states including neurodegenerative disorders like Alzheimer's (Benuck et al. 1996; Averna et al. 2001). Two important isoforms of this type, calpain I and calpain II are found in tissues, and remain as the well-characterized members of the family to date. These two isoforms need micromolar and millimolar concentrations of Ca^{2+} for in vitro activity, respectively (Goll et al. 2003). Calpains are involved in the neuronal pathogenesis of AD, where variables such as oxidative stress and enhanced instigation of the *N*-methyl-D-aspartate (NMDA) receptor are pivotally involved in the activity of calcium flux of intracellular region, thereby upregulating these enzymes (Ono and Sorimachi 2012). Experiments performed using AD culture model systems, revealed that oligomeric Aβ caused a substantial (fivefold) and immediate increase in Ca^{2+} in hippocampal neurons (Kelly et al. 2005; Kelly and Ferreira 2006, 2007).

Once activated, calpain triggers cleavage of p35 into its active form—p25 that interacts with CDK5, together with several other proteins such as, GSK3, DARPP-32 (Dopamine- and cAMP-regulated Phoshoprotein-32), increases BACE1 production, and truncates APP (Liang et al. 2010; Cho et al. 2015; Yan et al. 2016) as explained in the previous section. Sequentially, the calpain-mediated excision of DARPP-32 resulted in the dephosphorylation of a critical protein known as CREB (cAMP-response element-binding protein) engaged in cognitive and learning activities, as well as the conversion of immediate memory to long-term memory (Cho et al. 2015). Calpain also cleaves lysosome-associated proteins important for stability of lysosomes such as heat shock protein (Hsp) 70, and lysosome-associated membrane protein 2a (LAMP2a) (Arnandis et al. 2012; Yamashima 2016). Furthermore, calpain promotes ERK 1/2 activation and ERK 1/2-dependent phosphorylation of neurofilament protein residues observed to be altered in Alzheimer's disease. The cleavage of tau by calpain results in the formation of a 17 kDa toxic tau fragment, made up of the N-terminal half of tau protein (Park and Ferreira 2005). Calpain activation may potentially contribute to synaptic dysfunction via cleavage of PKA by calpain, resulting in reduction of the kinase's activity. The reduction in PKA

activity reduces CREB activation, thus impairing memory (Liang et al. 2010). Calpain interacts with a number of proteins and is an essential constituent of many AD-associated pathogenic pathways.

3.3.3 Dynamin-Related Protein 1 (Drp1)

Mitochondria contribute significantly to brain activity maintenance by supplying energy on a continual basis (Qi et al. 2019). Balanced mitochondrial dynamics are required for mitochondria to perform its usual tasks, such as performing oxidative phosphorylation and producing enough energy for neurons. Dysregulation of Drp1, Mfn1, Mfn2, and OPA1 proteins during mitochondrial fission and fusion subsequently leads to erratic mitochondrial dynamics (Tyumentsev et al. 2018). Abnormal Drp1 leads to the numerous structural deformities (small and slim, long and thin) in the mitochondria causing inadequate energy supply and ROS overproduction in neurons, resulting in a dysfunctional neural system and neurodegenerative disorders (Qi et al. 2019). Mitochondrial coordination failure is a prominent early characteristic in vulnerable neurons of an AD patient's brain. This is characterized by mitochondrial fragmentation, aberrant mitochondrial distribution, and mitochondrial malfunction, which leads to neurodegeneration (Wang et al. 2017).

Researchers discovered a link between a mitochondrial protein—dynamin-related protein 1 (Drp1) and Aβ. Studies based on fluorescence methods have shown a preferential contact between Drp1 and phosphorylated tau, which enhances the connection between Drp1 and Aβ, resulting in increased mitochondrial fragmentation (Yang et al. 2021). Drp1 association with Aβ monomer and oligomer, has been shown by immunoprecipitation/immunoblotting analyses of AD brain using Aβ antibodies 6E10 and A11 (Manczak et al. 2011). Further, Drp1 interaction with glycogen synthase kinase 3 (GSK3) has been prominently observed in the AD pathophysiology (Sayas and Ávila 2021). GSK3 is a Ser/Thr kinase present in almost all the tissues, it exists in two isoforms GSK3α and GSK3β, out of which the latter one is crucially present in the pathophysiology of AD (DaRocha-Souto et al. 2012). Drp1 protein is essential for the mitochondrial fission/fragmentation process via its GTPase activity. GSK3β activates Drp1 via phosphorylation of Ser40 and Ser44 leading to elevation of proapoptotic mitochondrial activity and fragmentation (Rippin and Eldar-Finkelman 2021). More research is needed to infer whether mitochondrial dynamics may be used as a therapeutic target for neurodegeneration.

3.3.4 BACE-1

BACE1 (β-site APP cleaving enzyme I) is a type I transmembrane aspartyl protease explicitly expressed in the brain, especially in neuronal cells, oligodendrocytes, and astrocytes (Hampel et al. 2021). BACE1 is found at the healthy synaptic terminals, the plasma endothelial membrane, and endosomal sections. BACE1 is a β-secretase enzyme essential for Aβ genesis, as it cleaves the transmembrane APP to dissociate

the β-stubs, and is proved to be the rate-limiting step in Aβ production (Vassar 2004; Sathya et al. 2012). Once β-secretase cleaves APP at the Asp+1 and Glu+11 site, the soluble N-terminal domain of APP gets released, while the C-terminal domain (CTF-β or C99) stays attached to the membrane, where it has been shown to impair synaptic functioning, thus aggravating AD symptoms (Tamayev et al. 2012). The excision of Aβ is a sequence-guided process as proven by several site-directed mutagenesis and radio-sequencing experiments (Haass et al. 1995). Hydrophobic substitutions of amino acids surrounding the excision site in APP are known to increase or decrease the efficiency of β-secretase cleavage. For instance, point mutations of methionine present in the APP's P1 region to heavier hydrophobic amino acid such as Leu resulted in elevated activity of BACE-1, while Met-Val mutants are resistant to BACE-1 cleavage (Haass et al. 1995; Cole and Vassar 2007).

BACE1 concentrations and activity were shown to be elevated in human AD brain extracts, which are in coherence with the experimental findings that neurons express greater amounts of Aβ in AD than in "cognitively healthy aging" (Kandalepas et al. 2013). Furthermore, a relatively significant concentration of BACE1 was observed in neurotic dystrophies around Aβ plaques in both AD amyloidogenic transgenic mice models and in the brains of AD patients, suggesting that its occurrence may enhance cyclic Aβ formation (Sadleir et al. 2016). BACE1 is considered a potential target for AD treatment, since this enzyme essentially limits reaction rates in the amyloid pathways (Das and Yan 2017). An uncommon mutation in APP, specifically at the human BACE1 cleaving site, results in a 40% reduction in the production of Aβ, hence decreasing the propensity of Aβ to aggregate. This mutation results in five- to-sevenfold reduction in aggregation of Aβ fibrils lowering the chance of AD development, and better cognitive performance in older individuals. As a result, BACE1 inhibition is expected to benefit AD patients (Das and Yan 2019). However, BACE-1 is a structurally complicated protein due to its high structural similarities with other aspartic proteases, large active site (consisting of the catalytic aspartic residues, flap, and 10S loop) which has been an influential hindrance in design and development of BACE-1 inhibitors. Despite such hurdles, several potent inhibitors of BACE-1 protein have been developed and are comprehensively discussed in a recent review (Moussa-Pacha et al. 2020).

3.3.5 Munc18-Interacting (Mints) Proteins

Munc18-interacting proteins (Mints/X11s) are the members of an adaptor protein family produced by *abpa1, abpa2,* and *abpa3* genes. They encode neuron-specific Mint1 (X11α) and Mint2 (X11β), as well as universally expressed Mint3 (X11γ) (Sullivan et al. 2014). The N-terminal portion of Mint proteins is variable depending on the isoform, while the C-terminal region is conserved and includes a PTB (phosphotyrosine binding) domain along with two tandem PDZ (PSD-95/discs large/zonula occludens) domains in the C-terminal of Mints (Xie et al. 2013). All three Mint proteins contain a PTB domain that binds to the YENPTY motif of APP

that is functionally conserved, and is significantly required for the trafficking of APP and influences the Aβ synthesis (Müller et al. 2017).

Several studies specified that the APP–Mint interaction is predominantly significant in AD. According to the findings of Ho et al. in mice models of AD, loss of each unique Mint isoform slows down the development of age-dependent Aβ plaques (Ho et al. 2008). In addition, Mint proteins interact with presenilin-1, the catalytic domain of γ-secretase composite, through their PDZ domains, boosting the localization of APP/presenilin-1 simultaneously, which favors the development of Aβ plaques (Sullivan et al. 2014). Furthermore, Mint proteins have observable role in the formation of neurotic plaques and have been observed to be elevated in AD (McLoughlin et al. 1999). In a recent study, Bartling et al. demonstrated that Mint2-specific protein–protein interaction with APP facilitates the formation of Aβ (Bartling et al. 2021). Collectively, these outcomes recommend that the APP–Mint interaction could be beneficial in the management of Aβ plaque development.

Various knockout in vivo experiments, have been published in an attempt to understand the physiological function of Mint proteins in Aβ production (Guénette et al. 2017). However, these investigations showed that Mint proteins had both inhibitory and facilitative effects on the generation of Aβ plaque. The function of Mint proteins in the development of pathogenic Aβ will remain as a mystery until the successful identification of chemical tests that disturb the APP–Mint PPI are discovered.

3.3.6 Chemokines

Chemokines are chemotactic cytokines that were first discovered as mediatory cues of immune cell migration to inflammatory sites. Chemokine receptors, which belong to the GPCR superfamily, are highly expressed in the central nervous system (Rossi and Zlotnik 2000). The chemokines show affinity toward multiple receptors that initiate the kinase-based signaling cascade (Tripathi and Poluri 2020). Chemokines are divided into CXC, CC, CX3C, and C sub-families based on the order in which the first two cysteine residues appear at the amino-terminal region of the protein (Bachelerie et al. 2013). In case of Alzheimer's disease, inflammation is one of the major changes in the physiological state of the neural system. The elevated inflammatory response is highly connected to the increased expression of chemokines and their receptors, as shown in plasma, cerebrospinal fluid, and brain tissue of Alzheimer's patients (Liu et al. 2014). Chemokines and their receptors are believed to be produced primarily by microglia, astrocytes, and neurons and most of them are involved in the neuroinflammation of AD through glial cell activation and by employing peripheral blood monocytes (Martin and Delarasse 2018). Chemokines belonging to the CXC family such as CXCL8, CXCL10, and CXCL12 are also responsible for the development and advancement of AD. CXCL8/CXCR2 and CXCL12/CXCR4 axis are reported to be elevated in the neurons, microglia, and astrocytes in response to the proinflammatory signals. These chemokines are also involved in the modulation of synaptic transmission and neuronal excitability (Jorda

Table 3.3 Alteration in the level of chemokines majorly observed in the patients with Alzheimer's disease

Sl. No.	Chemokine	Expression levels		
		Serum	Brain tissues	CSF
1.	CCL2	Upsurged	Upsurged	Upsurged
2.	CCL3	NA	Upsurged	NA
3.	CCL5	Reduced	Upsurged	NA
4.	CXCL10	NA	NA	Upsurged
5.	CXCL12	Reduced	NA	Reduced
6.	CXCL8	Upsurged	Upsurged	Upsurged
7.	CX3CL1	Reduced	NA	NA

CSF, cerebrospinal fluid; *NA,* not available

et al. 2020). Various chemokines having pivotal role in progression of AD and alteration in its expression leading to progression of Alzheimer's are tabulated in Table 3.3.

Moreover, overexpression of CC chemokines like CCL2 and CCL5 are reported in the brain, mature senile plaques, microglia, and microvessels of AD patients, suggesting that they are detrimentally involved in the disease development (Haskins et al. 2016; Zhou et al. 2016). Clinical evidence from Alzheimer's patients shows enhanced level of these chemokine/chemokine receptors in both CSF and plasma, thus corresponding with disease progression and cognitive deterioration (Zhang et al. 2013). It is also evident from a transgenic knockout mouse model of AD that these chemokines interact with their cognate receptors, and induce the Aβ and tau protein pathogenesis in due course of AD progression (Zuena et al. 2019). Recently in a mouse-based study, Joly et al. established that overexpression of CCL2 enhances the pathogenic tau protein (τ-p) that activates the harmful neuroinflammatory changes in the glial cells (Joly-Amado et al. 2020). Overexpressed monocyte chemoattractant protein 1 (MCP-1), well-known as CCL2 has been identified in the brain, mature senile plaques, microglia, and microvessels of AD patients, thereby implying it necessitates a detrimental role in the disease's development (Vukic et al. 2009). Clinical evidence from Alzheimer's patients has shown an increase in CCL2 in both CSF and plasma, according to researchers, this corresponds with disease progression and cognitive deterioration (Zhang et al. 2013). It is evident from an in vivo model of AD (Tg2576 mice and APPswe/PSEN1) that CCR2 deficiency in mice triggers macrophage recruitment, microglial buildup, and Aβ clearance (Zuena et al. 2019). Lately, Joly et al. in a murine model study, also established that overexpression of CCL2 is related to the development and progression of AD (Joly-Amado et al. 2020).

3.4 Parkinson's Disease (PD)

Parkinson's disease is a multifactorial nervous system ailment that has a negative impact on motor function. The symptoms begin gradually, with a hardly perceptible tremor in only one hand. People may have difficulty walking and talking as the disease progresses (Schapira et al. 2017). This disease affects over ten million people universally, and is one of the most dominant neurodegenerative ailments, second only to Alzheimer's. It primarily affects people over the age of 60 and worsens over a period of years, but early onset of Parkinson's disease can affect people as young as 30 (Rizek et al. 2016).

Parkinson's disease exhibits a distinguished loss of nerve cells in the substantia nigra region of the brain. This part of the brain's nerve cells is responsible for producing a chemical called dopamine. An amalgamation of genetic, environmental, and age-related factors is believed to contribute to dopamine-producing nerve cell demise, which in turn affects the body's capability to move and function. PD is associated with the cytosolic inclusion called Lewy bodies, whose primary constituent is aggregated α-synuclein (Rodriguez et al. 2015). Despite our current limited knowledge regarding the exact cause and molecular mechanism of α-synuclein aggregation, it has been proposed that its defective interaction with other protein partner leads to its aggregation. Molecular mechanism underlying this aggregation and related pathological aspects of the disease are dependent on the changes in this interaction (Gómez-Benito et al. 2020). The study of α-synuclein interactions with its partner proteins can help researchers to better understand the pathogenesis and discover novel pharmaceutical targets for Parkinson's disease therapy.

3.4.1 α-Synuclein

α-synuclein (α-S) is a *snca*-encoded small protein of molecular weight ~15 kDa, because of its propensity to aggregate, this protein has a unique pathogenic characteristic (Fan et al. 2021). Its structure is comprised of two alpha helices, which are trailed by unstructured, acidic C-terminal fragments (Fig. 3.3). Because of the repeated KTKEGV motif, multipole α-helical structure develops, the initial alpha-helix is charged positively and interacts with lipids, whereas the second alpha-helix comprises of a hydrophobic nonamyloid beta component (NAC) responsible for its aggregation (Du et al. 2003). The protein C-terminus is extremely acidic and contains ten glutamate and aspartate residues which is the reason of its exceptional thermostability. It is also demonstrated to control the accumulation of synuclein; fragments having shortened C-terminal aggregate quicker than the full-length protein. It is also evidenced that the PTMs like C-terminal phosphorylation, affect the aggregation tendency of α-S (Brown and Horrocks 2020). Under physiological circumstances, α-S belongs to the family of intrinsically disordered proteins (IDPs), as it has possessed no permanent structure. Rather, it shows transition amongst a set of constantly interchanging conformational states. Some of these conformations become stable when attached to other proteins or lipid membranes.

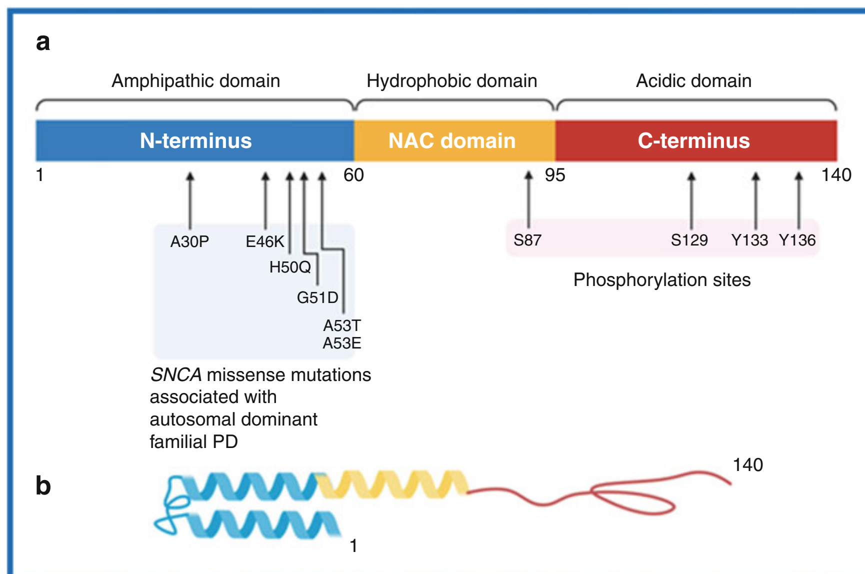

Fig. 3.3 (**a**) Structural architecture of the α-synuclein monomer representing the known mutations and phosphorylation sites associates with the progression of Parkinson's disease. (**b**) 3D representation of the α-synuclein monomer. (Adapted from Fan et al. 2021)

For instance, many investigations have demonstrated that α-S assumes an α-helical conformation when attached to phospholipid membranes of natural occurrence or produced synthetically (Gambin et al. 2011).

α-Synuclein establishes many interactions with other partner proteins throughout their biogenesis to ensure correct structural and functional aspects. Most of these interactions take place cotranslationally at the time of protein synthesis in the ribosome (Hernandez et al. 2020). As seen on many other proteins, altering these connections may interfere with protein synthesis and contribute to a variety of human illnesses, including neurodegenerative disorders. Several studies have shown that any malfunctioning of α-S interactions, especially at the time of translation will result in the misfolding and aggregation of the protein that ultimately, cause the development of disease. Aggregation of the protein may be triggered by a forfeiture of support through α-S biogenesis caused by an alteration in the gene of α-S or by the removal of an associated partner protein from the system. Protein–protein interactions with α-S may occur at both the cotranslational and post-translational stages. The embryonic chain is by now uncovered to a range of interacting partners at an early level of translation, which include chaperones/chaperonins, translocating and targeting factors, modifying enzymes, and a variety of additional partners (Karamyshev and Karamysheva 2018). In a recent study, researchers proposed that there are various proteins interacting with the α-S during its process of biogenesis; Fig. 3.4 highlights the predicted PPIs in the α-Syn-mediated pathology. In further sections of this chapter, numerous important

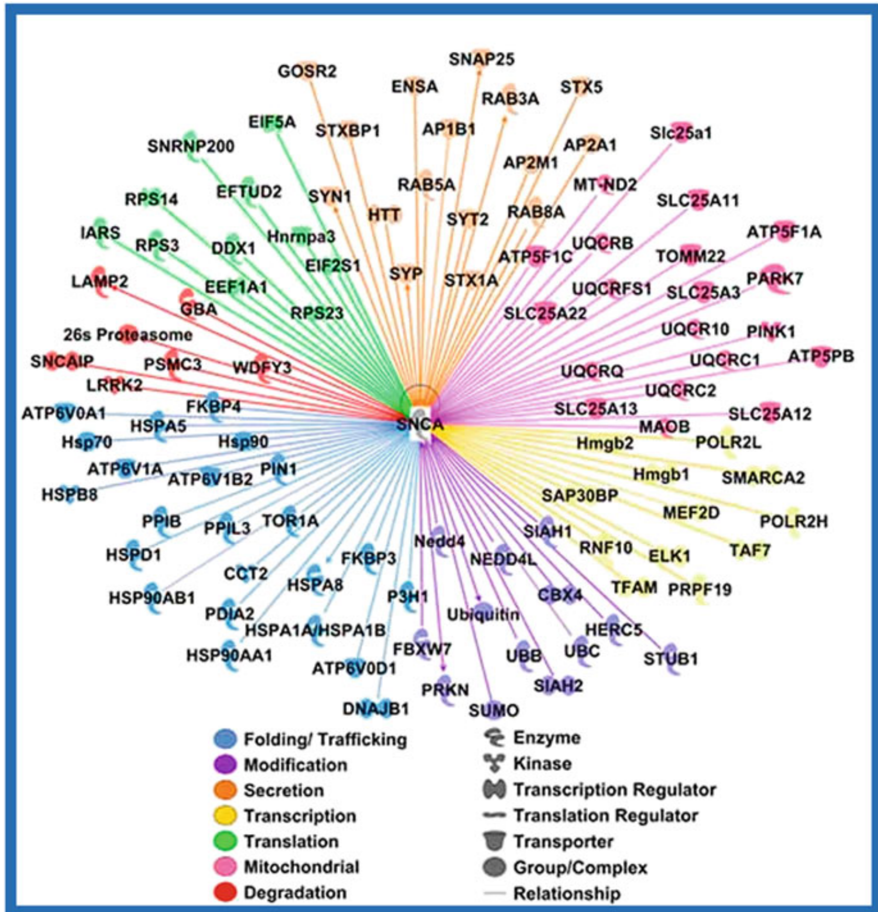

Fig. 3.4 Predicted PPI in the pathology of α-Syn generated using ingenuity pathway analysis (IPA) provided by Qiagen. (Adapted from Hernandez et al. 2020)

proteins that crucially participate in the initiation and progression of the PD have been discussed.

As a result of finding the genetic variants of PD, it has become clear that the autophagy/lysosomal and mitochondrial/oxidative stress pathways are critical in the pathological aspects of the disease. Newly discovered PD-linked genes, such as *sh3gl2* (endophilin A1) and *dnajc6* (auxilin) have revealed that disintegrations in synaptic vesicle endocytosis (SVE) are noteworthy influencers of disease pathogenesis in addition to other factors. Other Parkinson's disease genes, such as *lrrk2, prkn*, and *vps35,* are also identified as fundamental in the regulation of SVE. Nguyen et al. have highlighted the influence of dysfunctional SVE to midbrain dopaminergic neurons' selective susceptibility, as well as pathways that establish the relationship

of synaptic, mitochondrial, and lysosomal malfunctioning in the pathological aspects of Parkinson's disease (Nguyen et al. 2019).

3.4.2 Endophilin-A1 (*sh3gl2*)

Endophilin-A1 (EP) is a protein specific to brain and localized predominantly in the synaptic terminals (Micheva et al. 1997). It is observed to be involved in the release of neurotransmitters by interacting with synaptojanin, synaptotagmin, SNAP25 (synaptosomal-associated protein 25), and vesicle glutamate transporter 1. Moreover, EP is also potentially involved in the process of synaptic vessel endocytosis (SVE), which is a crucial phenomenon for the removal of neurotransmitters from synaptic clefts along with the morphogenesis and stability of dendritic spines (Yang et al. 2015). Endocytosis of synaptic vesicles is facilitated by the association of EP with synaptojanin, which is essential for the retrieval of synaptic vesicles. As a result, EP is an extremely important molecular player in the regulation of synaptic transmission (Schuske et al. 2003).

Despite the fact that EP plays a critical part in synaptic transmission, only a handful of studies in the last decade have demonstrated that EP can act as an intermediary of synaptic dysfunction in neurodegenerative diseases. It is evidenced that EP is crucially involved in the synaptic malfunctioning and neuronal injury in Parkinson disease (Zou et al. 2021). Heutink and Verhage had an intriguing finding that the EP associates with leucine-rich repeat kinase 2 (LRRK2) and parkin, where it serves as a substrate for phosphorylation or the ubiquitination, resulting in synaptic dysfunction in patients with PD (Heutink and Verhage 2012). *sh3gl2* gene (gene-encoding endophilin A1) was recognized as a PD risk factor in a large-scale GWAS meta-analysis and was found to significantly control the synaptic vesicles' endocytosis. Furthermore, in the recent years it has been established that not only the variant of *sh3gl2*, but also variations in the binding domain of its microRNA can lead to the commencement of PD. Additionally, previous research had long established that *sh3gl2* plays a significant role in the normal functioning of CNS, and any abnormalities might result in the onset and development of Parkinson's disease (Nguyen et al. 2019). Although, the physiological utility of endophilin A1 in neural terminals is well recognized, its definite molecular mechanism in the etiology of Parkinson's disease remains unknown.

3.4.3 Cathepsin

As discussed in the previous section, α-syn is proven to be related to PD both genetically and neuropathologically (Lashuel et al. 2013), and understanding the molecular processes that govern α-syn aggregation is crucial in developing strategies to slow the advancement of Parkinson's disease. Impaired α-syn turnover is among the many of the key causes of Parkinson's disease (Rubinsztein 2006). It is well established that lysosomes and proteasomes are engaged in the degradation of α-syn

aggregates (Wong and Cuervo 2010). The proteasome pathway eliminates the soluble α-syn, and eradicates aggregation-prone species or excessive amounts of soluble α-syn. It has been demonstrated that dysfunction of either system causes a rise in α-syn levels (McGlinchey and Lee 2015). α-syn reaches lysosome by one of the two primary autophagic pathways: macroautophagy or chaperone-mediated autophagy. Once the protein is present in the lysosome, it is destroyed by the proteasome (Xicoy et al. 2019). The cathepsins are the most important family of lysosomal proteases, and they are further classified depending on the amino acids in the active region that are responsible for catalytic activity. They can be classified as cysteine (CtsB, CtsC, CtsF, CtsH, CtsK, CtsL, CtsS, CtsV, and CtsX) proteases, serine (CtsA and CtsG) proteases, and aspartyl (CtsD and CtsE) proteases (Turk et al. 2012).

Recent research shows that inflammatory responses displayed by glial cells, such as the proliferation of T cells, and augmented expression of inflammatory cytokines are now recognized as evident characteristics of PD. In addition, some of the toxic intermediaries derived from activated Glial cells are also observed in due course of diseases (Pišlar et al. 2018). Along with the inflammatory cytokines, microglial cells in their activated state also release cathepsins, which accelerate neurodegenerative mechanism and assist the process of neuronal death in the Parkinson's diseases (Pišlar and Kos 2014). Although foregoing research suggests that cathepsin D (CtsD) is critically responsible for the development of PD, the issue whether additional lysosomal proteases are also involved has been raised. For instance, in vivo evidence shows the significant link between CtsD overexpression and α-syn concentration, and the neurotoxic potential of such interaction (Cullen et al. 2009). CtsD is speculated to participate in the partial lysosomal disintegration of α-syn, specifically in generation of truncated C-terminal (α-syn) species (Sevlever et al. 2008). The α-syn C peptides, particularly polypeptides like 1–87, 1–103, and 1–119 of α-syn (140 residues), exhibit higher prospect of amyloid formation in the acidic lysosomal lumen habitat. While the occurrence of α-syn C species is low under physiological circumstances, some of these α-syn species (1–115, 1–119, 1–122, 1–133, and 1–135) have been extracted from Lewy bodies, which are characteristic of PD (McGlinchey and Lee 2015).

3.4.4 Parkin (PARK2)

Parkin is a 52 kDa protein synthesized by the *park2* gene and comprises of 12 exons and 465 amino acids. Mutation in *park2* gene leads to the autosomal recessive type of PD called early-onset PD (EOPD) observed in various ethnic groups (Hedrich et al. 2004). Parkin is an E3-ubiquitn ligase involved in a variety of neuroprotective functions, such as maintaining mitochondrial metabolism and the ubiquitin-proteasome structure, where parkin is required for the ubiquitin-arbitrated disintegration of misfolded or damaged proteins as well as the degradation of dysfunctional mitochondria via mitophagy (Geisler et al. 2010; Giguère et al. 2018). Parkin mRNA has also been found in abundance in the tissues of heart and skeletal muscle,

indicating that the protein is extensively distributed throughout the body. Parkin catalyzes mono- and poly-ubiquitylation of a variety of structurally and functionally different proteins, including itself (Sarraf et al. 2013). Over 100 mutations (including missense mutation, significant chromosomal deletion or duplication, truncation, and promoter alterations) in 12 exons of PARK2 gene have been found as being linked with Parkinson's diseases. Apart from these mutations, post-translational modifications (such as S-nitrosylation, covalent dopamine interaction, kinase-mediated phosphorylation) and oxidative stress are also reported to be involved in blighting the activity of parkin, resulting in the development and progression of sporadic Parkinson's disease (Madsen et al. 2021).

α-Synuclein endures wide-ranging post-translational modifications, mainly phosphorylation and nitration. Cumulative evidences are there to support the fact that these PTMs, are necessary for the aggregation of α-synuclein and neurotoxicity in PD (Zhang et al. 2019). An in vivo experimental model unraveling the consequence of phosphorylation of α-synuclein and involvement of parkin gene on α-synuclein aggregation exhibited that, due to the increase in the glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation of α-synuclein and tau, cell death and attenuation in inflammation take place (Khandelwal et al. 2010). Apart from phosphorylation, all the tyrosine residues of α-synuclein undergo nitration. Neuroinflammation of PD is associated with a nitric oxide synthase (NOS)-mediated increase in the generation of nitric oxide (NO), facilitating the formation of neurotoxic oligomers in neurons (Stone et al. 2012). Aggregated α-synuclein and tau in neurofibrillary tangles are collective hallmarks of PD and Alzheimer's disease respectively (Lee et al. 2001). Albeit these two proteins are involved in two entirely different diseases, there is a pile of experimental evidence which support the fact that both proteins mutually accelerate the mechanism of PD (Moussaud et al. 2014). It is observed that α-synuclein mediates the phosphorylation of tau protein by stimulating the activity of protein kinase A (PKA) (Qureshi and Paudel 2011). Similarly, GSK3β is an enzyme that hyperphosphorylates the tau protein at its serine residues and enhances the neurotoxicity in PD (Duka et al. 2009). Further, studies also establish that this serine phosphorylation is inhibited by the PD-linked parkin protein. It is observed that the loss of neuroprotective functions of parkin leads to the misfolding and aggregation of α-synuclein (Madsen et al. 2021).

A major effector of parkin in progression of PD is PINK1 protein. Parkin together with its interacting PTEN-induced kinase 1 (PINK1) are essential in maintaining a healthy mitochondrial network by stimulating the process of mitophagy, and is an important therapeutic target for Parkinson's disease. According to the current model, in healthy state (mitochondria with intact potential), PINK1 enters the mitochondria and is degraded by PARL (presenilin-associated rhomboid-like protein). But under diseased state, the degradation of PINK1 does not take place and it accumulates on the outer mitochondrial membrane (OMM), where it phosphorylates ubiquitin at the Ser65 of OMM proteins resulting in recruitment of parkin on the OMM. The recruited parkin further phosphorylates PINK1 and stimulates the ubiquitination of OMM proteins such as mitofusin 2 and VDAC1 (voltage-dependent anion channel 1) creating a feed-forward mechanism. The presence of ubiquitin on the OMM

proteins prompts the introduction of ubiquitin adapter proteins such as NDP52 which promotes mitophagy (Miller and Muqit 2019). Several studies have been conducted to evaluate the approaches targeting the parkin-mediated response during Parkinson's disease, and have been reviewed in detail elsewhere (Senkevich et al. 2022).

3.4.5 Apolipoprotein E (APOE)

Apolipoprotein E is a 35 kDa protein synthesized majorly in the liver and brain. This protein is crucial for fat metabolism and maintenance of cholesterol homeostasis in plasma and CNS of mammals. This protein is also significantly involved in the cognitive function of the human brain (Mahley and Huang 2012). In general, there are three alleles of this protein, namely, ε2, ε3, and ε4 positioned on 19q chromosome (Verghese et al. 2011). The ε4 is the most harmful of the three common APOE alleles, followed by, ε3 and ε2 alleles (Raichlen and Alexander 2014). In addition to the plasma, APOE is important for the maintenance of cholesterol homeostasis in the central nervous system (CNS). The maintenance of normal neuronal growth, membrane plasticity, and synapse formation is dependent on balancing the proper cholesterol homeostasis. Hence, a shortage of cholesterol in the brain results in poor neural plasticity and decreased neurotransmission, which ultimately result in brain damage. APOE produced by astrocytes is entirely brain-specific, and there is no interaction between plasma-derived APOE and brain APOE because of blood–brain barriers (BBBs) (Bales 2010). Along with the abovementioned proteins showing direct or indirect interactions and their deleterious effects in PD, there are various other proteins as tabulated in Table 3.4 which are observed to be involved in PD as well.

3.5 Amyotrophic Lateral Sclerosis (ALS)

ALS, often called as Lou Gehrig's disease is a degenerative ailment of the nervous system that destroys the cells of spinal cord and brain leading to the unregulated behavior of muscles. Common symptoms of ALS include twitching of muscles along with limb weakness and incoherent speech/slurred speech. ALS eventually impairs muscular control, affecting the ability to move, talk, eat, and breathe. This deadly condition has no known treatment (van Es et al. 2017). Characteristic features of ALS are progressive degeneration of motor neuron, not only in motor cortex, but also in brain stem and spinal cord. Pathology is assumed to originate at a focal area within the CNS, and is linked to the deposition of proteins into insoluble inclusions, as is the case with other neurodegenerative illnesses (Fatima et al. 2015). Inclusions linked with ALS may occur in the surrounding oligodendrocytes and astrocytes, in addition to cell groups beyond the pyramidal motor system. Histopathological investigations have revealed that, like other neurodegenerative ailments and prion

Table 3.4 Summary of the major proteins involved in Parkinson's disease

S. No.	Protein name	Description	Localization	References
1.	Endophilin-A1 (SH3GL2)	Involved in synaptic vesicle endocytosis.	Cytoplasm, presynaptic vesicle	Nguyen et al. (2019)
2.	Parkin (PARK2)	Catalyzes the interaction of proteins-ubiquitin moieties in a multiproteic complex (E3 ubiquitin ligase). Regulate the protein breakdown.	Membrane	Zou et al. (2021)
3.	Leucine-rich repeat kinase 2 (LRRK2)	Interact with C-terminal of PARK2 protein and regulate its activity.	Cytoplasm, mitochondria-l membrane	Zou et al. (2021)
4.	Apolipoprotein E (APOE)	Facilitating the transport of cholesterol and other fats through the circulation by regulating the intermediates in the catabolism. Linking, and internalization of lipoprotein particles.	Cytoplasm	Emamzadeh (2017)
5.	Vesicle-fusing ATPase; (NSF)	Responsible for the intracellular transport of protein. Involved in the arbitration of protein catabolism and recycling of receptors.	Golgi bodies, cytosol, and membrane	Belluzzi et al. (2016)
6.	AP2-associated protein kinase 1 (AAK1)	Control the clathrin-arbitrated endocytosis by phosphorylating the adaptor protein complex 2 (AP-2). It is also involved in regulating the vesicle transport.	Presynaptic vesicle, membrane	Kostich et al. (2016)
7.	Cyclin-G-associated kinase (GAK)	Associated with the functioning of cyclin G and CDK5. Involved in the HSC70-mediated uncoating of vesicles having clathrin coating.	Golgi bodies, and membrane	Dumitriu et al. (2011)
8.	Huntingtin-interacting protein 1-related protein (HIP1R)	Involved in the actin binding. Crucial for the cell survival through receptor stabilization and endocytosis induced by the ligand.	Membrane and perinuclear region	Teixeira et al. (2012)
9.	Tropomyosin α-1 chain (TPM1)	Bind specifically to the actin filament. Crucial for the contraction and relaxation of the muscles.	Cytoplasm	Botelho et al. (2020)
10.	Semaphorin-5A (SEMA5A)	Regulate the bifunctional axonal guidance signal through interaction with sulfated proteoglycans like heparan	Membrane	Bossers et al. (2009)

(continued)

Table 3.4 (continued)

S. No.	Protein name	Description	Localization	References
		sulfate and chondroitin sulfate. Involved in the regulating the focal adhesion disruption in glioma cells.		
11.	Cathepsins	Cathepsins are thiol protease responsible for the intracellular degeneration of neuronal and glial cells.	Lysosome, ER	Li et al. (2011)

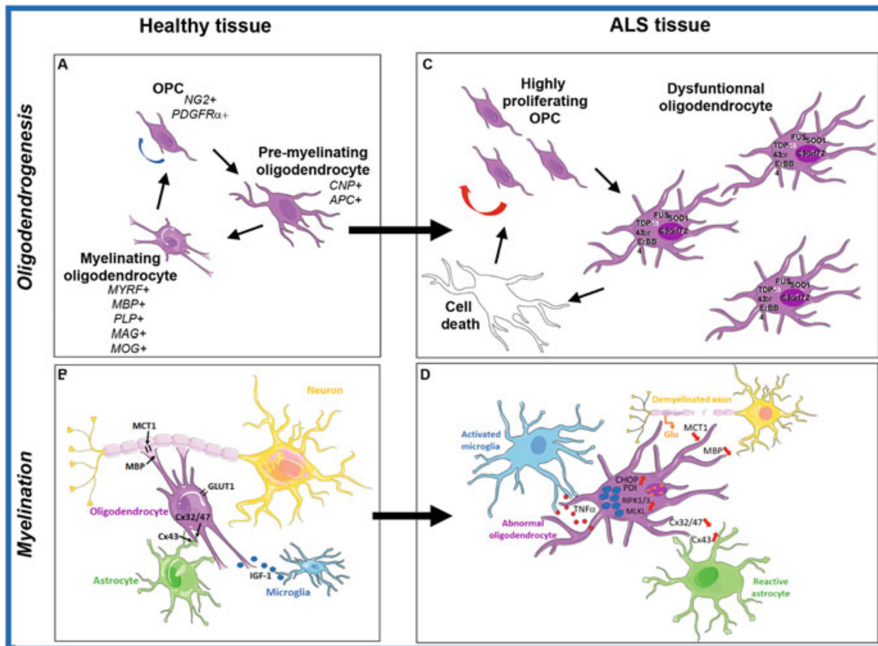

Fig. 3.5 Pathogenesis of amyotrophic lateral sclerosis (ALS). (**a**, **b**) In a healthy neuronal cell, the process of oligodendrogenesis and myelination. The oligodendrocyte progenitor cells (OPC) differentiate and mature into oligodendrocytes and myelinating cells that facilitate the transport of lipids and other nutrients for the production of myelin sheath. (**c**, **d**) In an ALS condition, differentiation of OPC is upsurge that gives rise to the aggregates of the various proteins like FUS, TDP-43, and SOD-1. In addition to this, the unusual oligodendrocytes fail to mature in myelinating cells that leads to the axonal demyelination. (Adapted from Traiffort et al. 2021)

disorders, pathology spreads from a focal spot across linked cells in phases (Brettschneider et al. 2014).

The crucial proteins that are allied with the pathology of ALS are; transactive response DNA-binding protein 43 (TDP-43), superoxide dismutase-1 (SOD1), and fused in sarcoma (FUS) (Fig. 3.5) (Arai et al. 2006; Vance et al. 2009). A

considerable proportion of ALS patients (about 97%) are observed to have the inclusion of TDP-43, with the remaining instances being correlated to either SOD1 (approximately 2%) or FUS (approximately 1%) inclusions, respectively (Ling et al. 2013). TDP-43 protein binds to the DNA or RNA and is crucially involved in the number of metabolic activities of RNA. On the similar note, FUS protein also possesses the DNA/RNA-binding features along with the RNA metabolism. It is involved in the DNA-damage response and acts as a transcription factor as well (Wang et al. 2013). SOD1 is one among the major antioxidant enzymes in the cell, and it is responsible for converting superoxide anion into less toxic molecular oxygen species that are less detrimental to the cell (McCord and Fridovich 1969). Furthermore, TDP-43, SOD1, and FUS are proficient in generating amyloid fibrils in vitro (Nomura et al. 2014), and there are compelling evidences to indicate that they adopt an amyloid morphology in vivo, at least in the cases of SOD1 and TDP-43 (Bigio et al. 2013; Robinson et al. 2013). Despite the fact that mislocalization of TDP-43, phosphorylation, and protein aggregation in motor neurons is the most common clinical signature of ALS, only a small number of individuals have TDP-43 mutations in their cells. Most of the ALS cases are sporadic (sALS), as is the case with most other neurodegenerative illnesses including prion disorders that account ~90% of all instances of ALS, whereas the other instances are familial (fALS). Although some of the fundamental hereditary origins of the illness are still unknown, they are often connected with a history of the disease in the patient's family (Taylor et al. 2016).

3.5.1 TAR DNA-Binding Protein-43 (TDP-43)

The *tardbp* gene on chromosome 1 encodes a 414 amino acid-long TDP-43 protein that is mostly found in the nuclear region, although it also moves to the cytoplasmic region to perform certain functionalities (Ayala et al. 2008). TDP-43 plays varied roles pertaining to RNA metabolism; it is responsible for transporting and migrating the mRNA, miRNAs, and lncRNA (Coyne et al. 2017). TDP-43 acts together with many other proteins involved in diverse physiological activities, as shown by a global interactome analysis (Freibaum et al. 2010). Blokhuis et al. recently analyzed the interacting protein partners linked to the ALS pathogenesis in the neuronal cells, which led to the identification of numerous DNA/RNA-binding proteins in the TDP-43 interactome. These proteins are observed to be crucially regulating the processing of RNA, expression of required gene, RNA splicing, apparatus of post-transcriptional gene expression such as translational and post-translational modifications (Blokhuis et al. 2016). In ALS, the cytoplasmic TDP-43 concentration rises, resulting in the creation of cytoplasmic inclusions. TDP-43's entire structure has remained elusive so far because of its low in vitro solubility and strong aggregation tendency (Winton et al. 2008; Wang et al. 2016). However, many groups have identified highly resolved structures of several of its domains, though overall structure of this protein is still unresolved. TDP-43 interacts with various

proteins directly or through RNA-dependent interactions, and some of the major interactions are extensively summarized recently by Prasad et al. (2019).

TDP-43 proteinopathies are characterized by nucleocytoplasmic mislocalization, aggregation of ubiquitinated and hyperphosphorylated TDP-43 in the inclusion body, and accumulation of toxic truncated C-terminal fragment of TDP-43 in the brain region. Mutations (sporadic or familial) may exacerbate these deleterious possessions, and result in commencement of the diseases at an early age. Variety of *tardbp* mutants/variants have been demonstrated as being related with Lou Gehrig's disease (Buratti 2015). Greater proclivity of this protein to aggregate increases the possibility of cytoplasmic mislocalization, changed protein stabilities, protease resistance, and abnormal association with other proteins. These are some of the major consequences of these mutations in the TDP-43 protein. Indeed, the mutations at the carboxy-end of the TDP-43 protein increase the protein's innate aggregation tendency. The recombinantly generated TDP-43 mutant protein with ALS-associated mutations including Q331K, M337V, Q343R, N345K, R361S, and N390D has enhanced aggregation in vitro and boosted cytotoxicity (Johnson et al. 2009). The mutations in the TDP-43 protein have the potential to disrupt the protein's stability, which could be a plausible pathogenic mechanism. On similar note, it was established that the TDP-43 protein mutant (G298S, Q331K, and M337V) showed a longer half-life and greater stability in an isogenic cell line as compared to the wild-type TDP-43 (Ling et al. 2013). Other mutations also enhance the vulnerability of TDP-43 to protease-assisted degradation, which is another important pathological aspect of ALS (Nonaka et al. 2009). The consequence of TDP-43 mutations and their role in the pathology of ALS have been extensively discussed elsewhere (Mitsuzawa et al. 2018).

Furthermore, the deletion of TDP-43 in nucleus and its accumulation in cytoplasm are also a very crucial factor to be considered in the ALS pathogenesis. TDP-43 binds to a number of nuclear proteins that are responsible for the splicing of mRNA along with the metabolism of other RNA. In addition, it also interacts with the cytoplasmic proteins that are responsible for the translation of mRNA (Ling et al. 2010). The NLS-NES (nuclear localization signal and nuclear export signal) of TDP-43 control the nucleocytoplasmic exchange of TDP-43 (Winton et al. 2008). Mutation at any of this signal sequence would lead to the aggregation of TDP-43 in the nuclear region. Apart from the mutations, the two foremost prevalent PTMs in protein TDP-43 that are associated with its pathogenic significance are phosphorylation and ubiquitination (Arai et al. 2006; Hasegawa et al. 2008). Subsequently, additional PTMs such as acetylation, poly ADP-ribosylation, and cysteine oxidation were also found in the patients with ALS. Comprehensive portrayal of the PTMs holds the possibility of uncovering new harmful TDP-43-mediated pathways in ALS (Kametani et al. 2016).

3.5.2 Superoxide Dismutase-1 (SOD1)

Among some of the reported genes responsible for ALS, the *sod1* gene continues to be a prominent source, and has been the subject of numerous research (Tafuri et al. 2015). The gene responsible for making the SOD1 enzyme, a 32 kDa homodimer in its native state that holds the feature of binding with copper and/or zinc ion. Structurally, SOD1 enzyme constitutes a metal-binding site, a disulfide bond, along with a network of hydrogen bonds that hold each SOD1 subunit's architectural structure together (Banci et al. 2002). The metal-binding site of SOD1 contains two copper ions and one zinc ion, that are responsible for its catalytic activity. This protein is majorly located in the cytosolic region of the cells, along with the nucleus, peroxisomes, and mitochondria, among other places (Estácio et al. 2015). It has been established that, point mutations in *sod1* gene may cause abnormality in its structure that induces malfunctioning of this protein leading to ALS. More than 180 point mutations in human SOD1 have been discovered, with over 160 linked to ALS (Pansarasa et al. 2018). SOD1 mutant protein may adopt a variety of misfolded conformations that specifically induce the altered features of ALS.

The evolution of SOD1 comprises numerous phases and is quite complicated. The proliferative phase of SOD1 protein generally occurs in the cytoplasmic region. Through mutation-based research, pathogenic mutations are postulated to postpone the maturation of the protein that is expected through post-translational modifications, lower the structure-based stability of protein that consequently promotes the SOD1 protein misfolding (Furukawa et al. 2016). ALS-associated mutations of SOD1 may induce a change in arrangement leading to gain of toxicity, although the fundamental mechanism is only partially known (Atlasi et al. 2018). Further, it has been established that the misfolded SOD1 interacts with the protein involved in mitochondrial dysfunction, gene transcription, it also has the interacting partners related to the endoplasmic reticulum (ER) stress. Moreover, it has been documented that RNA processing is one of the crucial aspects of central dogma, and mutated SOD1 is reported to be interacting with protein-inducing abnormal RNA processing (Huai and Zhang 2019).

Concentration of misfolded SOD1 increased on mitochondrial cytoplasmic face through its adherence in a straight line to a voltage-dependent anion channel (VDAC1) and/or Bcl-2. VDAC1 is a transmembrane protein that establishes channels which govern ion and metabolite flow across the mitochondrion, allowing it to communicate with the rest of the cell. The explicit interaction of SOD1 mutants to VDAC1 decreases its conductance, reducing VDAC1 activity and decreasing SOD1 mutant mouse survival (Israelson et al. 2010). Bcl-2, a mitochondrial outer membrane protein, promotes cell viability by blocking proapoptotic proteins. When associated to a SOD1 mutant, Bcl-2 undergoes a conformational shift that exposes its deadly BH3 domain (Pedrini et al. 2010). Furthermore, SOD1 mutations have been shown to enhance the interaction with ER-resident membrane protein Derlin-1, causing the death of motor neuron in turn directing to ALS etiology (Tsuburaya et al. 2018). Derlin-1 protein is responsible for the ER-associated degradation (ERAD) process, it is well-documented that a communication between SOD1

mutations and Derlin-1 causes the stress in the ER region due to the failure of ERAD, which results in ASK1 stimulation and death of motor neurons, thus dictating the course of ALS illness (Nishitoh et al. 2008).

Vascular endothelial growth factor (VEGF), which was first identified as a crucial factor regulating the process of angiogenesis, is now recognized as a growth factor possessing neuroprotective features that are vital for the survival of motor neurons. In murine model, it has been explained that the reduced expression of VEGF genes resulted in a phenotype that is comparable to individuals with ALS. These findings, indicate that the damage of VEGF is associated with the pathophysiology of PI3K/Akt signaling in ALS and motor neuron degeneration (Pronto-Laborinho et al. 2014). SOD1 mutants have been demonstrated to be linked to the VEGF 3′--untranslated region (UTR) through competition with the ubiquitous RNA-binding and stabilizing protein (RBP) HuR, with a preference for adenine/uridine-rich regions (ARE), resulting in a decrease in the stability of VEGF mRNA and a decrease in VEGF expression (Lu et al. 2009; Srikantan and Gorospe 2012).

The ubiquitin-proteasomal system (UPS) along with the autophagy-lysosomal systems are the two primary protein degradation mechanisms in eukaryotic cells. In ubiquitinated proteins, proteasomes preferentially remove surplus or broken peptide links through proteolysis (an organic event that disrupts the peptide bonds). In contrast, autophagy allows for the controlled breakdown and reutilization of accumulated proteins and cellular constituents (Huai and Zhang 2019). The SOD1 mutation associated with ALS has been shown to interact directly with the S6 (Rpt3/PSMC4) and S6′ (Rpt5/PSMC3) domains of the 19S monitoring complex of the proteasome, impairing the standard activity of SOD1. Additionally, the SOD1 mutant tangibly binds with the CHIP (Hsc70-interacting protein) a co-chaperone, as well as the ubiquitin-binding proteins such as Bag1 and valosin-containing protein/p97 (Choi and Lee 2010). Interestingly, CHIP and VCP contend for interacting with the mutant SOD1, implying that the chaperone complex (CHIP/Hsp70) and the proteolytic apparatus (VCP/26S proteasome) are in a competition to bind to the same substrate. CHIP acts as a connecting factor among chaperones and UPS and presumably controls the equilibrium of the protein refolding and degradation as well (Choi and Lee 2010; Lin et al. 2013).

3.5.3 Fused in Sarcoma (FUS)

Fused in sarcoma (FUS) is a 57.8 kDa (526 amino acid) oncogenic DNA/RNA-binding protein which is also associated with neurodegenerative disorders. In human liposarcoma, the FUS gene was described as a fusion oncogene situated on chromosome 16 (Crozat et al. 1993). FUS is a crucial part of the major cell functioning, that includes regulation of gene transcription, DNA repair, RNA shearing and its transport, translation, and maintaining the stability of genome (Lagier-Tourenne et al. 2010). Under typical physiological circumstances, FUS is mostly found in the neuronal and glial nucleus; however, being an RNA-binding protein, it also participates in nucleocytoplasmic transport (Brelstaff et al. 2011).

FUS-linked ALS is majorly because of the mutations in the FUS gene; almost 50 mutant FUS genes have been discovered in patients with ALS ailment. The majority of them are missense mutations, with a handful having in-frame deletions. Numerous FUS mutations, notably the most frequent mutant R521C is specific to humans, arise inside the greatly conserved C-terminal nuclear localization signal (Deng et al. 2014). Particularly, certain mutations, such as P525L, are linked to a condition of extra severe advancement of diseases that is typically fatal before it can be passed (Kent et al. 2014; Nolan et al. 2016). Mutations with ALS are often seen to be causing disruption in the nuclear localization signal of FUS, resulting in cytoplasmic mislocalization and subsequently the development of aggregates. Several animal models clearly imply that FUS induces motor neuron degeneration by inducing a cytoplasmic deleterious gain-of-function, although it is possible that a decrease in nuclear FUS contributes to the disorder (Dormann et al. 2010). In several ALS models, RNA binding is essential for complete FUS toxicity to manifest itself. The detailed characterization of both the cytoplasmic and nuclear RNA interaction networks of FUS is therefore, essential not only for an improved understanding of its physiological function, but also it can offer additional valued understanding of the molecular mechanisms that underlie neurodegeneration in ALS, which are currently unknown.

3.6 Huntington Disease

Huntington's disease (HD), well-known as Huntington's chorea, is a genetically inherited autosomal neurodegenerative illness marked by progressive motor dysfunction, cognitive weakening, and dementia (Pandey and Rajamma 2018). The medium spiny neurons of the caudate putamen are the principal sites of neurodegeneration, which worsens in a dorsomedial to ventrolateral direction, although the region's interneurons are mostly spared. The afflicted areas of the brain are cerebellum, thalamus, and white matter widen as the severity of the illness worsens. The development of the illness is usually around midlife, and the sickness might last for decades (Vonsattel et al. 2011). Expansion of CAG repeats encoding glutamine on gene *it-15* occurs in HD, this expansion leads to the abnormal long glutamine tract at the N-terminal of huntingtin proteins (HTT), and this is most prevalent among the other nine poly-glutamine diseases. A critical glutamine level of ~37 and above, acts as a marker of HD that induces agglomeration of mutant HTT in insoluble neuronal "inclusion bodies" and specific brain degeneration in the cerebral cortex and striatum region (Estevez-Fraga et al. 2020). Symptoms of HD may appear in the patients of all the age group, but usually in midlife and it takes 10–15 years for initiation, progression, and to reach the chronic stage of the disease. HD is a typical protein-misfolding ailment, where enhanced polyQ tract disturbs the native protein conformation resulting in worsening of the symptoms. Based upon the serial MRI of asymptomatic and symptomatic patients of HD, it is observed that HTT protein expresses and causes pathological stress throughout the brain (Kloeppel et al. 2009).

A large number of research evidenced that the mutation in HTT exerts its pathological effect in a prevailing manner, also the expansion in polyQ tract is observed to have the cytotoxic characteristics leading to the misfolding and aggregation (Neueder et al. 2017). Although majority of HD affected people have one copy each of normal and mutant *IT-15,* it is assumed that the dominance of mutant HTT is because of the impaired protein interactions that cause toxicity and eventually lead to the neurodegeneration seen in HD (Pandey et al. 2018). The N-terminal region of HTT protein comprises of polyQ stretch which gets cleaved off in small fragments to interact intracellularly and facilitate the aggregation of HTT protein (Estevez-Fraga et al. 2020). Recently, researchers observed that mutating the N-terminal region averts the aggregation of proteins but only the development of large aggregates is prevented, but not the small oligomers. Moreover, the toxicity in the HD is augmented proving the fact that the large aggregates are not significantly involved in neural death (Branco-Santos et al. 2017). Furthermore, mutated HTT is also observed to be involved in hindering the cellular responses like heat shock response and mitochondrial unfolded protein response (UPR), required for refolding of proteins and also in activating the proteasomal machinery for the degradation of misfolded proteins (Hipp et al. 2012) as diagrammatically summarized in Fig. 3.6.

It is crucial to illuminate the interaction cohorts of both wild type and mutant HTT for better understanding the pathological machinery of HD and standard functioning of HTT. To serve this objective, Goehler et al. studied the protein–protein interaction systems for HD and observed various uncharacterized new proteins (Goehler et al. 2004). These protein partners have been observed to be involved in pathological machinery of mutant HTT as well as in cellular processes such as transcription of gene, endocytosis, trafficking of vesicles, and signal transduction initiated by wild-type HTT (Li and Li 2004). From the available data, it has been proposed that, the complexity in the pathology of HD might be ascribed due to the partial or complete loss/gain of these varied PPIs. In the following subsections, some of the crucial protein partners that are associated with the development and progression of HD are discussed.

3.6.1 Transcription Factors

Transcriptional dysregulation is a well-known pathogenic mechanism in HD. Numerous transcriptional proteins are observed to be interacting with both wild type and mutant HTT proteins, and these proteins are also observed to form an interrelated group with each other. Because of its polyglutamine expansion, mutant HTT has an aberrant interaction with numerous proteins that are involved in transcriptional control (Valor 2015). Some studies stress upon the significance of PPIs between mHTT and other transcription factors, coactivator, or other transcriptional regulators, while others emphasize direct mHTT–DNA binding as a causative mechanism implicated in transcriptional malfunction in HD pathobiology (Zhai et al. 2005). These groups of protein consist of the global transcription controller TATA-binding protein ($TF_{II}D$), $TAF_{II}130$, a coactivator associated with cAMP-responsive

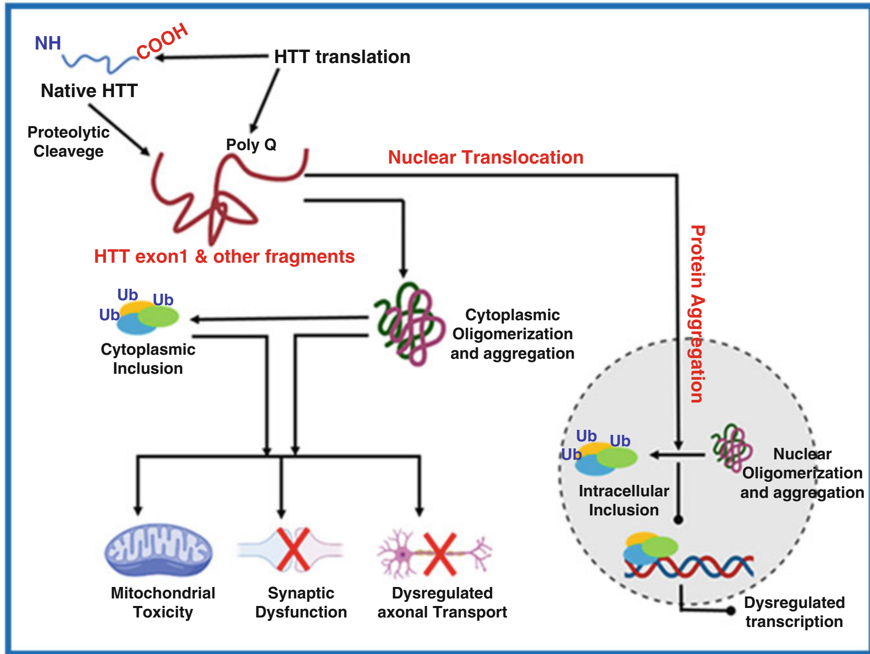

Fig. 3.6 Pathophysiology of Huntington diseases (HD). Post-translation, *htt* gene develops a full-length HTT protein along with an N-terminal HTT exon-1 fragment that is produced due to the dysregulated splicing. Extra fragment of cleaved proteins will be produced by the proteolytic cleavage of full-length HTT. These fragments of proteins are translocated to the nuclear region, where it aggregates and leads to the formation of inclusion body that hampers the process of transcription by interacting with the transcription-regulating proteins. In addition, HTT proteins also aggregate in the cytoplasmic region, which affect the functioning of other organelles and lead to universal cellular impairments that include mitochondrial toxicity, dysfunction in the synaptic transmission, and unregulated axonal transport

element-binding protein (CREB)-dependent transcription (Moumné et al. 2013). Specificity protein 1 (Sp1), p53, CREB-binding protein (CBP), and nuclear receptor corepressor (NCoR) also demonstrated to have an abnormal interaction with mutant HTT (Wanker et al. 2019). It has been shown that the polyglutamine mutation by themselves are not able to degrade the glutamine-rich cellular transcription factors; while the soluble form of mHTT can easily disrupt the interface between the transcription factors (for example, Sp1) and transcriptional coactivator (like transcription initiation factor $TAF_{II}130$) along with their target DNA.

CBP acts as a coactivator for a diverse number of transcription factors that include CREB and Sp1. An abnormal association between mHTT and glutamine-rich region of CBP disrupts its coactivator activity, which sequentially hampers the normal function of related transcription factors (Glajch and Sadri-Vakili 2015). CBP's histone acetyl transferase (HAT) activity is also disrupted, along with its coactivator function that results in the cognitive loss as seen in HD (Giralt et al. 2012). The

transcriptional repressor element-1 restrictive silencer factor (REST) is a corepressor that suppresses the production of target genes such as brain-derived neurotrophic factor (BDNF) and miRNAs. Standard cytosolic HTT protein binds to REST with high affinity and blocks its nuclear migration, and therefore positively regulating the expression of its target genes (Bithell et al. 2009). In contrast, the mHTT affinity for REST is lower, resulting in increased nuclear entry, and eventually the deregulation of known REST target genes such as BDNF and miRNAs. Certainly, BDNF appearance has been observed to be impaired in HD models (Zuccato et al. 2001). In addition to this, HTT also interacts with p53, and as a result, HTT may have an impact on the transcription of p53 target genes, which include the genes responsible for the regulation of cell cycle, stress responses, cell death, and DNA repair (Bae et al. 2005).

3.6.2 Autophagy-Related Protein

Autophagy (also known as macroautophagy; MA) is a lysosomal degradation system that is characterized by the production of an autophagosome, a double-membraned vesicle that separates the cytoplasmic contents for destruction. The autophagosome eventually unites with a lysosome to produce an autolysosome, which degrades the contents. Autophagy has been shown to be changed in NDDs in many studies, and increasing autophagy has been recommended as a possible treatment method (Martin et al. 2015). Autophagy is important for maintaining brain homeostasis through selective protein degradation, in addition to removing toxic and misfolded proteins and in case of NNDs, as the change in autophagy eventually affects the neuronal activities. After a number of studies revealing autophagy abnormalities in HD; for instance, PolyQ-HTT via inactivating the mammalian target of rapamycin (mTOR) kinase, inappropriately stimulated the autophagy pathway in several HD models. Despite the fact that autophagosome synthesis is unusually high in HD, there is a deficiency in autophagosome loading, resulting in a diminished ability of cells to digest aggregated proteins and organelles (Steffan 2010).

HTT may control selective autophagy directly via a variety of ways. Firstly, HTT modulates the reverse transport of autophagosomes through axons, by acting as a scaffold for the dynein/dynactin/HAP1 complex. HTT silencing decreases optineurin localization in the Golgi apparatus, suggesting that HTT regulates autophagosome/lysosome dynamics through association between HTT and optineurin/Rab8 (Wong and Holzbaur 2014). Secondly, the domains of HTT are remarkably similar to those of the yeast autophagy proteins Atg23, Vac8, and Atg11. HTT's C-terminal region, which is comparable to yeast Atg11, interacts with the mammalian homologs of the Atg1/ULK1 kinase complex, while its central domain that is similar to Vac8 is found to be associated with Beclin-1 (Ochaba et al. 2014).

In addition, the existence of 11 LC3-interacting repeats (LIRs) in HTT adds to the evidence that HTT plays a function in selective autophagy. LIR motifs like these can be found in Atg8 family-associated proteins like p62 and optineurin. They are

important for autophagy receptor protein ability in cargo attachment to LC3 and/or GABA-receptor-associated protein (GABARAP). The discovery of HTT's p62-interaction domain shed light on the methods by which HTT controls cargo recognition as well as autophagy induction. HTT binds to p62, making it easier for p62 to recognize ubiquitinylated proteins at Lys63, and allowing cargo to be loaded onto autophagosomes (Rui et al. 2015). Furthermore, upon association of HTT with ULK1, mTOR-mediated phosphorylation of ULK1 is inhibited, thus resulting in autophagy activation. HTT's autophagy regulating role is preserved in flies and can be fine-tuned by changing the 3D structure of the protein. Indeed, the C-terminal domain of HTT, which is comparable to Atg11, binds with an extra N-terminal region of HTT, which is similar to Atg23, resembling the yeast Atg11–Atg23 interface (Ochaba et al. 2014).

Ultimately, differences in the ratio of polyQ within the normal range may influence HTT function in autophagy. In mice, removing the typical Q stretch stimulates autophagy and extends their lifespan (Zheng et al. 2010). HTT aids p62-mediated cargo identification during autophagy, at least in part, by increasing the affinity between p62 and ubiquitylated cargos and LC3. Due to a failure in cargo identification, such a functionality is disrupted in HD, resulting in the creation of empty autophagosomes. HTT loss or polyQ expansion in HTT, both result in HTT's decreased role in autophagosome formation in such a scenario (Rui et al. 2015).

3.6.3 Cell Division-Related Proteins

HTT is prevalently involved in the process of cell division, in the areas containing high number of microtubules such as astral microtubules, mitotic spindles, and spindle poles that are not confined to differentiated neurons. HTT is guided to the spindle poles during mitosis of neuronal as well as nonneuronal cells through its interaction with dynein, where it stimulates the gathering of NUMA and LGN (Godin et al. 2010). Likewise, HTT also controls the trafficking of dynactin, mitosis regulation proteins such as nuclear mitotic apparatus1 (NUMA), and Gα-binding protein (GPSM2/LGN) besides astral microtubules to the cell cortex in mammary gland cells through a dynein facilitated kinesin-1 mechanism. This cortical localization of dynein-dynactin complex, NUMA, and LGN is vital for mitotic spindle orientation by generating pulling pressures on astral microtubules. As a result of the HTT malfunctioning during mitosis, the misorientation of spindles is observed (Elias et al. 2014). Meanwhile, neurons in HD brains have been demonstrated to reenter the cell cycle upon the expression of the mHTT gene. Cell signaling pathways that are critical for cell division, neural development, and neuroprotection are in particular abnormally regulated in HD. Assessing the potential relationship among mature neuron dedifferentiation and aberrant stimulation of other brain cell types, especially neuroblasts and glial cells, in alliance with distorted cytokine signal transduction in pathological brains may thus provide legitimate clues for identifying potential therapeutic targets for the treatment of NDDs (Manickam et al. 2020).

3.6.4 Trafficking Vesicle-Associated Proteins

HTT connects with the molecular motor machinery perhaps directly with dynein or indirectly with the p150-glued component of dynactin. In addition, the KIF5C, a member of kinesin 1 family also interacts with HTT through the Huntingtin-associated protein 1 (HAP1) (Twelvetrees et al. 2010). These linkages help HTT in controlling organelle movement in both the anterograde and retrograde orientations, as well as in neuron axons and dendrites. Although the exact details of the molecules translocated by HTT are still unknown, it has been found to be associated in trafficking of synaptic precursor vesicles, vesicles with the VAMP7 protein, autophagosomes, endosomes, and lysosomes, brain-derived neurotrophic factor (BDNF), APP-positive vesicles, and GABA-receptor-containing vesicles. The efficient vesicle movement is dependent on the HTT overexpression (Saudou and Humbert 2016).

It is worth mentioning that BDNF transport is decreased to the physiological level in knock-in mice with mutant HTT (mHTT), irrespective of the allelic zygosity. Similarly, BDNF transport is decreased in neurons of heterozygous HD patients' human embryonic stem cells (hESCs). Remarkably, silencing the mutant allele reestablishes the BDNF transport to regulate hESC levels, indicating that silencing mHTT selectively may reduce the negative influence of dominant mHTT on wildtype HTT-regulated vesicular function (Drouet et al. 2014). Therefore, mHTT loses its capacity to boost BDNF vesicular transport in HD, but operates dominantly on wild-type HTT to change transport. These examples establish how an aberrant polyQ expansion might affect HTT functions in a variety of ways (Saudou and Humbert 2016).

3.7 Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a genetic disorder distinguished by muscle wastage (atrophy) and weakness (skeletal muscles). It is initiated by the loss of alpha motor neurons in the spinal cord, which are specialized nerve cells that govern muscular movement (Kolb and Kissel 2011). When comparing muscles near the center of the body (proximal) to the muscles farther distant from the center of the body (distal), the weakening is more acute in the proximal muscles leading to paralysis in chronic condition. Muscle weakness frequently develops as people become older. Werdnig and Hoffmann published the first description of the condition in the 1890s (Hoffmann 1892; Werdnig 1971); in 1995, the survival motor neuron (*smn*) was identified as the disease-initiating gene, allowing the genetic abnormality to be pinpointed to the 5q11.2-q13.3 region of the genome (Lefebvre et al. 1995). SMA is one of the most prevalent pediatric recessive hereditary illnesses, and condition is divided into four phenotypes: SMA I: never achieve unassisted sitting; SMA II: unassisted sitting; SMA III: unassisted walking; SMA IV: adult onset, based on the beginning age and the greatest developmental motor milestone reached as summarized by Prior et al. (2020). SMA type I (SMA-I)

reports for 50–60% of all diagnosed SMA cases, and is the most severe type of SMA that frequently results in mortality before the age of two. Type II, III, and IV of SMA are among the less severe variations (Mercuri et al. 2020). Muscle atrophy, weakness, as well as eating and breathing problems, are major indicators of this deficiency.

SMA is triggered by a deficit of the universally expressed survival motor neuron (SMN) protein, which is caused by homozygous deletion or, less typically, minor alterations of *smn* gene. On chromosome 5q13, there are two nearly similar *smn* genes (*smn1* and *smn2* genes) that determine spinal muscular atrophy that differs by a single-nucleotide (C-T) change. Such alternation although does not affect the arrangement of amino acids, it causes exon 7 to be spliced differently (Wirth et al. 2020). Owing to variation in alternative splicing of exon 7, *smn2* genes generate a truncated and unstable protein. In individuals, the muscular dystrophy majorly occurs due to transformation of *smn1* to *smn2*, while in carriers of SMA deletion of a *smn1* allele and minor intragenic alterations have been observed (Calucho et al. 2018). Hence the severity of the illness is mostly determined by quantitating the *smn2* transcripts and the truncated SMN protein as shown in Fig. 3.7. Usually in the case of SMA type I, carriers retain two copies of *smn2*, while in the case of SMA type II, three copies of *smn2* prevalently exist, and similarly, in the case of SMA type III and IV, three or four copies are generally preset in respective cases (Kolb and Kissel 2011). The SMN protein is involved in a number of physiological functions that harbor protein–protein interactions. Some of the relevant interactors of SMN and their details of the interaction are discussed as follows.

3.7.1 Plastin 3 (PLS3)

In mammals, along with the SMN2 copies, the severity of SMA can also be greatly affected by the MN-independent protective modifier genes. PLS3 is a protective gene modifier that elevates the severity of the SMA, and is found close to the DXZ4 microsatellite, which is critical for X-chromosome inactivation (Bonora et al. 2018). In SMA-discordant families, differential transcriptome analysis revealed up to 40-fold PLS3 overexpression in lymphoblastoid cell lines, but not in the fibroblasts of asymptomatic women. Notably, when these fibroblasts are reprogrammed into induced pluripotent stem cells (iPSCs) followed by their differentiation into MNs, the level of PLS3 doubles in asymptomatic women (Heesen et al. 2016). PLS3 SNVs or *cis*-regulatory elements linked to the asymptomatic phenotype have yet to be unraveled; PLS3 overexpression can only be tested at the RNA or protein level. Besides, the unexpected instigation of osteoporosis in humans and animals due to loss-of-function mutations in PLS3 is also observed (Neugebauer et al. 2018).

It has been further noted that the activity of PLS3 toward SMA severity is age and sex-specific (Alrafiah et al. 2018). An interesting association between PLS3 and SMN1-associated disease progression has been observed in various studies indicating inverse correlation between PLS3 and SMA severity. Although SMN controls the PLS3 protein levels, PLS3 concentration when restored can rescue

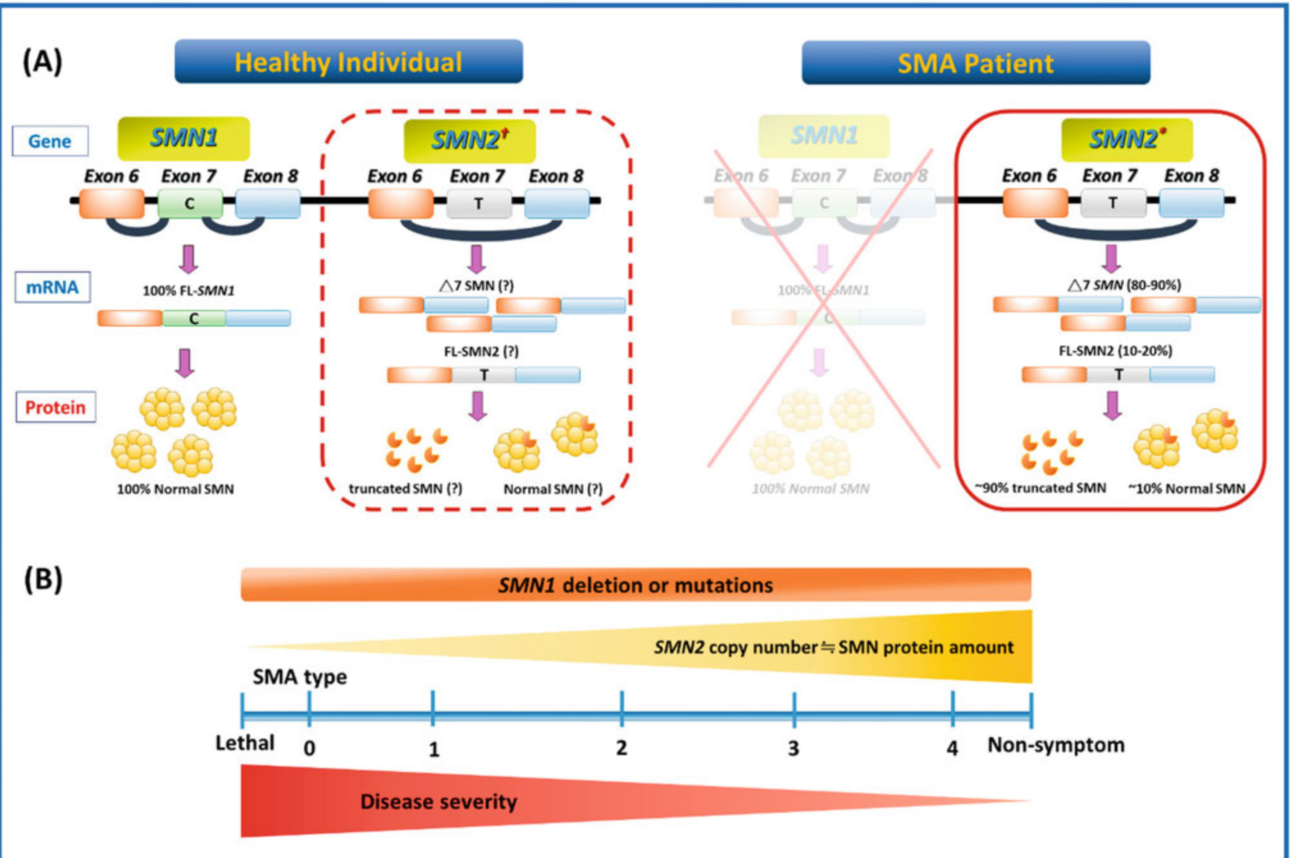

Fig. 3.7 Genetic coordination of spinal muscular atrophy (SMA). (**a**) In contrast to the healthy individual, *smn1* gene is hemizygously deleted in the carrier of SMA. At the same time, at least one copy of the *smn2* is reserved. (**b**) Graphic representation of the severity of SMA depending upon the *smn1/smn2* gene alteration. (Adapted from Chen 2020)

presynaptic and motor defects as proved in case of *smn*$^{-/-}$ zebrafish model (Dimitriadi et al. 2010). PLS3 acts as a protective modifier of SMN, although it does not directly interact with the SMN protein (Singh et al. 2021; Oprea et al. 2008). Instead of PLS3-SMN direct interaction, PLS3 has been suggested to partake in rescue of SMA pathology by stimulating the accumulation of actin filaments that is important for axon development (Alrafiah et al. 2018). Although PLS3 is an SMN-independent protective modifier, several other proteins such as CHP1 and CORO1C (calcineurin EF-hand protein 1 and coronin-1C) assist PLS3 in restoration of actin dynamics, calcium homeostasis, and endocytosis, the majorly perturbed biological processes in spinal muscular atrophy (Wirth 2021). Several human pathology studies and animal model experiments have proven that compared to other SMN modifiers, PLS3 is one of the most plausible effectors of SMA pathology (Wirth 2021; Hosseinibarkooie et al. 2016).

3.7.2 Gemins

Gemins are a group of eight diverse proteins which form ribonucleoprotein (RNP) macromolecular complexes together with SMN and Strap/Unrip protein that influences the chaperoning of snRNPs crucial for pre-mRNA splicing. The SMN1 protein is a constituent of the SMN complex, an in charge of assembling small nuclear ribonucleoproteins (snRNPs), which are obligatory for pre-mRNA splicing (Li et al. 2014). Moreover, Gemins are also involved in the assembly and localization of the macromolecular complexes. A recent study has shown that mutations in both SMN and Gemin5 exhibit detrimental effects on the tissue regeneration process in zebrafish model. Out of all nine proteins (SMN, Gemin1–8, Strap, and Unrip), SMN and Gemin5 are vital in defining the activity of the RNP macromolecular complex (Pei et al. 2020). Gemin5 identifies the RNA arm of snRNPs with the help of highly conserved sequence and conserved motifs (WD-repeat motif) to initiate the binding of Gemin2 and the core protein of snRNPs—the SMN protein (Zhang et al. 2011; Lau et al. 2009). Owing to their crucial role in formation of SMN ribonucleoprotein complex, SMN–gemin interactions have been studied as a therapeutic target. However, the key structural feature of SMN–Gemin interaction and SMN macrocomplex are still elusive, and most of the research till date has been more focused on delineating the role of SMN in the SMA prognosis (Borg and Cauchi 2014).

3.7.3 Profilins (PFN)

Profilins belong to a small family of actin-binding proteins that may restrict actin polymerization while facilitating actin sequestration. In addition to the conventional actin dynamics, it has also been connected to a variety of other disciplines of cell biology that include membrane trafficking, signal transduction across membrane, synaptic architecture, nuclear emigration, and splicing of mRNA (Pernier et al.

2016). Furthermore, recent literature unraveled the linkage of profilin isoforms to the initiation and/or advancement of the significant ailment of nervous system, recognized as spinal muscular atrophy (SMA). The phenomenon was studied by mutations in their specific ligands and in the profilin isoforms itself (Walter et al. 2021). SMN is well recognized for its involvement in RNA splicing, where it helps to assemble and guide tiny nuclear riboproteins (snRNPs). They are crucial for a wide range of cellular functions, including RNA metabolism, transport of mRNA, mRNA localization, cell signaling, and cytoskeletal dynamics. In general, the severity of SMA is determined by the alteration in the SMN1 and the corresponding SMN2 gene. Due to the absence of exon7, the SMN2 isoform is relatively unstable and cannot substitute for SMN1 completely. On the other hand, increased SMN2 protein levels can cause SMA to revert to its milder forms (Fallini et al. 2012).

The dynamics of cytoskeleton may be altered in SMA due to the interactions of SMN protein with cytoskeletal proteins. SMN1 interacts with both PFN1 and PFN2a; in which PFN2a is more tightly bound. SMN1 interaction with PFN2a is hampered by exon 5 or 7 deletions or missense mutations. According to the mapping of the SMN protein, the domain at the C-terminal having poly-proline is necessary for initiating their binding with profilins. Further, endogenous PFN1 and PFN2a colocalize with SMN in the neurite-like progressions of PC12 cells, and the nucleus of motor neurons (Murk et al. 2021). Moreover, numerous studies show that SMN has a direct regulatory role in profilin protein levels and function; splicing errors in the profilin gene have been discovered in SMN-deficient fission yeast. The SMN-facilitated splice defect reduces the level of profilin protein, causing the disruption of actin network homeostasis, culminating in disrupted cytokinesis, and endocytosis in fission yeast (Antoine et al. 2020).

3.8 Frontotemporal Dementia (FTD)

Frontotemporal dementia is a devious neurodegenerative disease characterized by gradual abnormalities in behavior, executive function, and language. After Alzheimer's disease and dementia with Lewy bodies, this condition is the third most common kind of dementia across all age groups, and it is a prominent cause of early-onset dementia (Vieira et al. 2013). Arnold Pick described the first patient with frontotemporal dementia in 1892; the patient exhibited aphasia, lobar atrophy, and presenile dementia. Alois Alzheimer recognized the typical relationship with Pick bodies in 1911, and termed the clinicopathological entity as Pick's disease, which led to the adoption of Pick's illness as a synonym for frontotemporal dementia. Clinical syndromes of FTD are classified into three types (Bang et al. 2015):

1. Variants in behavioral FTD (bvFTD), the most prevalent version marked by substantial behavioral abnormalities such as disinhibition, apathy, lack of empathy, stereotyped or obsessive behavior, hyperorality, and dietary alterations. On neuroimaging, frontal and/or anterior temporal atrophy can be seen, which is usually more severe on the right.

2. Nonfluent-agrammatic variant primary progressive aphasia (nfvPPA) is a medical condition categorized by agrammatism and dialogue difficulties. It is associated with left inner frontal and insular atrophy.
3. Semantic variant primary progressive aphasia (svPPA) is a diseased condition marked by the loss of object and word identification as well as bilateral anterior temporal atrophy.

FTD is considered to be inbred in an autosomal dominant pattern in around 40% of cases, and about 10% of these patients have a favorable family history. Within the variations, bvFTD and nfvPPA are the ones that are most likely to be inherited, and svPPA is the one that is in all probability to be sporadic. The same basic gene abnormality may affect both men and women (Deleon and Miller 2018). Rademekers and coworkers have discussed that, in a condition, where, FTD has hardly been recognized in previous generations, and has a high incidence of misdiagnosis, it might conceal a familial history of the condition. Furthermore, the medical phenomenology of FTD may vary even among the members of same family, making proper diagnosis and detection of a familial condition more challenging. Even if there is no evident history of FTD in the family, the doctor should consider referring the patient for genetic testing and counseling, since roughly 6% of patients with no reported case of FTD in the family have a genetic etiology (Rademakers et al. 2012). Different clinical manifestations might result from the same basic gene abnormality. In addition, age of beginning of disease, duration, locations, arrays of brain atrophy are some of the variables that may influence the phenotype of FTD in a variety of ways. Researchers also mentioned that, the progression of related medical characteristics such as Parkinson disease and psychiatric disorders may also add to the severity (Deleon and Miller 2018; Miller and Guerra 2019).

There is an aberrant type of tau protein in the brain of around 50% of persons with FTD, and approximately 50% of people with FTD have TDP-43 protein buildup. FUS protein accumulation affects a tiny number of people, roughly 5%. This interferes with normal cell functions and may result in cell death. In addition, mutation in the GRN gene encoding progranulin protein, VCP gene encoding Valosin-containing protein, CHMP2B gene encoding chromatin-modifying protein 2B protein, *tardbp* gene encoding TDP-43 protein (observed in the case of ALS too), sequestosome 1 (*sqstm1*) gene encoding p62, a ubiquitin -associated protein etc., are observed to worsen the case of FTD. All these mentioned proteins and their interacting partners are explained in detail in the further sections.

3.8.1 Tau Protein

The protein tau is encoded by *mapt* gene and is important in maintaining the microtubule stability and assembly, as well as intracellular signaling. Tau mutations may cause illness via a variety of methods, including influencing tau alternative splicing, increasing tau aggregation in the cytoplasm, and microtubule instability owing to variation in tau phosphorylation. *mapt* was the one among the initial genes

discovered to be linked with FTD, for the first time, in 1994, Lynch et al. associated FTD in an autosomal dominant family to chromosome 17q21.2 (Lynch et al. 1994). Based on the mutation, there are different categories and sites of frontotemporal lobar degeneration-tau pathology in neurons and glial cells. Frontotemporal lobar degeneration-tau pathology is seen if there exists a mutation in *mapt* exon 10, while in its absence the 3R and 4R tau are observed in the nerve cells (Ghetti et al. 2015).

Similar to major NDD-associated proteins, tau is also involved in proper cytoskeleton assembly and localization. Specifically, it regulates the organization of microtubules (MT) in neuronal cells, whose activity greatly depends on the two-way trafficking of cytoplasmic cargo from axonal to dendritic peripheries. Several biochemical and mutation studies have confirmed that dysregulation of Tau-MT complex leads to disassembly of microtubules and incongruent cellular polarity and viability (Brunello et al. 2020). Tau protein is majorly localized in the axonal region of the neurons; however, it can also be found in other locations (plasma membrane, nucleus, mitochondria, synapses, and dendrites) at various timepoints during neuronal development and homeostasis indicating its multifaceted roles in the cells. Besides, MT regulation, Tau protein has been shown to be involved in synaptic development and signaling regulation via its interaction with nucleic acids and membrane receptors respectively (Goedert et al. 2021). Structurally, the Tau protein consists of three regions: the projection domain (consisting of N1 and N2 domain), the proline-rich region, and microtubule-binding repeat domains or MTBD (consisting of R1, R2, R3, and R4 domains). The projection domain is the N-terminal domain of the protein consisting of 1–150 residues, and is involved in the modulation of the MTBD–MT interaction (Matsumoto et al. 2015); the mid region is the proline-rich domain (residue 151–243), which is an intrinsically disordered region involved in interaction with nucleic acids, and secondary messengers (such as Src homology-3, Fyn kinase), and neuronal signal transduction (Koren et al. 2020; Wang et al. 2006). The Proline-rich region is the major region involved in the secondary functions of the tau protein. Lastly, the C-terminal (residue 244–441) comprises the MTBD region. The MTBD together with the proline-rich domain is a major binding hotspot for a variety of NDD-associated protein such as FUS, presenilin-1, TIA-1, and α-synuclein (Morris et al. 2011). Tau protein is considered as an intrinsically disordered protein due to its ability to acquire various structural states, and propensity of aggregate formation. Numerous biophysical studies such as circular dichroism, NMR, and cryo-EM have been used to unravel the conformational states of tau in its physiological, pathological, and microtubule-bound states (Kellogg et al. 2018; Avila et al. 2016).

In pathological conditions, Tau protein is able to aggregate owing to the presence of two hexapeptide (VQIVYK and VQIINK) regions present in the third and second MTBD repeats. Both regions are involved in the tau homodimerization process which acts as a nucleation step for Tau aggregation (Von Bergen et al. 2000; Fitzpatrick et al. 2017). Other than the hexapeptides, two cysteine residues of R2 and R3 also mediate the aggregation and formation of paired helical filaments of Tau fibrils (Fitzpatrick et al. 2017). Mutation studies have deciphered the role of the hexapeptides' regions and their nearby residues in the stimulation of fibril formation.

A widely known mutation P301L mutation that promotes tau aggregation has been found in some FTD patients. Similarly, insertion of proline in the hexapeptides has been shown to reduce fibril formation via distortion of β-sheet structures (Bulic et al. 2010). Similar to these, the critical MT-binding residues (K257, G272, P301, V337, R460) have been identified with the help of mutation studies (Liu and Gong 2008).

Post-translational modification specially, phosphorylation also plays an important role in regulation of MT, and other Tau-related signaling processes due to its Ser/Thr-rich sequences. The phosphorylation mediates the cell signaling capability of Tau, and the phosphorylated state is tightly regulated via kinases and phosphatases (Martin et al. 2011). Three types of kinases are mainly involved in the phosphorylation of the Tau protein namely, proline-directed Ser/Thr kinase (GSK3β, CDK5), nonproline-directed Ser/Thr kinase (DYRK1A, PKA, CK1), and Tyr kinases (Fyn, Src) (Martin et al. 2013; Rawat et al. 2022). Several of these kinases are also associated with the hyperphosphorylation of Tau and other proteins that are able to form neurofibrillary tangles. Hyperphosphorylation is one of the major steps in the deposition of Tau neurofibrillary tangles and reduction of Tau affinity toward MT (Sotiropoulos et al. 2017). Other than phosphorylation, acetylation at multiple sites of Tau MTBDs has also been identified as a detrimental modification. MTBDs are also rich in lysine residues and major Tau residues such as K280, K274, K281 have been suggested as the site for acetylation leading to progression of NDDs (Min et al. 2015). However, a recent study evaluated the protective role of acetylation of lysine present with the KXGS motifs (K259, K290, K231, K353) (Xia et al. 2021), suggesting the dual role of post-translational modification in the management of NDDs.

The wide spectrum of structural and functional information available for Tau protein has driven the development of therapies against NDDs. Till date, most of the therapies developed against FTD and other NDDs have been designed around Tau and amyloid fibrils. Tau inhibitors include Tau phosphorylation inhibitors, aggregation inhibitors, immunotherapies, and gene-silencing therapeutics. The current scenario of drug development and therapeutics involving Tau and other FTD-associated proteins has been comprehensively reviewed by Panza et al. (2020).

3.8.2 Progranulin

The progranulin protein-encoding gene, *grn* is located on 17q21.31. Progranulin found in neurons and active microglia acts as a growth regulatory factor that stimulates signal transduction, and is crucial for the physiological processes like development, wound repair, and inflammation (He and Bateman 2003). Progranulin is synthesized by the activated microglial cells, which adheres to sortilin-1 present on the surface of the neurons, gets internalized, and is transported to the lysosomes. Inside the neuronal cells, progranulin helps to break down proteins, especially TDP-43. To this point, there have been more than 100 *grn* mutations that are associated with age-related proteinopathy (Moore et al. 2020). Altered *grn* genes have been discovered in around 5% of patients with sporadic, and 5–15% of those

with familial FTD. Although progranulin-mediated FTD is autosomal dominant, surprisingly 90% of affected people show visible symptoms at the age of 70. Symptoms start to show up about 6–7 years after the disease's initiation (Galimberti et al. 2018).

Dominant nonsense and frameshift mutations largely affect the *grn* gene leading to the generation and degradation of premature mRNA, which suggests that *grn* haploinsufficiency is the cause of *grn*-linked neurodegeneration. A growing number of *grn* mutations cause the gene to be deleted. These mutations affect how the gene is translated, processed, and secreted. People with *grn* mutation have *grn* protein deficiency in their CSF (cerebrospinal fluid) (Fyfe 2020). *grn* mutation shows a lot of different phenotypes. The two most frequent disorders in this category are frontotemporal dementia and nonfluent/agrammatic variant PPA. People who have a *grn* mutation are more likely to have clinical signs of mild Parkinsonism, Alzheimer, and motor neuron disease in their bodies. Most of the time, *grn* mutations cause corticobasal syndrome, or progressive supranuclear palsy (Terryn et al. 2021).

Progranulin is also known to mediate neuronal survival and differentiation and acts as a neurotrophic factor. Clinical studies and assessment of gradual reduction of CSF in FTD patients with *grn* mutation, and in vitro studies with recombinant progranulin and granulin E-peptide have indicated that progranulin promotes the survival and neurite outgrowth in animal models (Kao et al. 2017). However, the knowledge of progranulin-associated receptor and its mechanism still remains elusive. Several receptors such as TNF receptors, sortilins, and ephrin type-A receptor 2 (EPHA2) have been identified as potent receptors of progranulin-mediated neurotrophic responses (Neill et al. 2016; Hu et al. 2010; Tang et al. 2011). Nevertheless, it has been difficult to reproduce the interactions specifically in case of TNF receptor using immunochemical assays and sortilins, as TNF receptors being trafficking receptors and are mainly involved in the anterograde and/or retrograde endosomal trafficking of progranulin to lysosome. Interestingly, of the three progranulin-associated receptors, a definite mechanism of neuroprotective effect for EPHA2 has been proposed recently. The proposed mechanism states that progranulin binds to the EPHA2, a cell-surface receptor that activates the tyrosine kinase activity of EPHA2 and its downstream AKT kinases (Neill et al. 2016).

Other than cell surface receptors, progranulins have been reported to form heterodimers with a functionally similar protein, prosaposin (Nicholson et al. 2016). Similar to progranulins, prosaposins are glycoproteins that are trafficked through sortilins to lysosome and promote sphingolipid hydrolysis (Zeng et al. 2009). Moreover, similar to progranulins, the loss-of-function mutants of prosaposins lead to the development of lysosomal storage disease known as sphingolipidoses. The stark structural and functional similarity of both the proteins makes them amenable for heterodimer formation, which helps in regulation of intracellular and extracellular progranulin levels (Chitramuthu et al. 2017). Moreover, prosaposins aid the transportation of progranulins to the lysosome, which is important for lysosome function and regulation. The localization of progranulins in lysosome has been linked to the formation of lysosome and cellular proteostasis. For instance, progranulins are associated with the regulation of TFEB transcription

factor, which is important for lysosome biogenesis. Similarly, cleaved progranulins (granulins) impair the degradation of TDP43, an essential factor in several NDDs (Zeng et al. 2009). The diverse role of progranulins in development and aging has made it an important therapeutic target. Small molecule inhibitors such as suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor is known to regulate the expression levels of progranulins; however, it is not a highly reliable treatment methodology as expression levels of progranulins are tightly regulated by epigenetic modifications. A comprehensive knowledge of progranulin expression, cleavage, and mechanism of action still needs attention to formulate precise therapeutic strategies (Terryn et al. 2021).

3.8.3 Valosin-Containing Protein (VCP)

The gene on chromosome 9p13.3 *vcp* encodes for the valosin-containing protein (VCP) is a part of the ATPase that is associated with various activities (AAA+) protein family, and is involved in biological processes such as cell cycle control, membrane fusion, and the ubiquitin-mediated proteasome degradation cascade (Yeo and Yu 2016). The first reports of frontotemporal dementia-associated VCP mutations were linked to a rare illness called inclusion body myopathy with Paget disease and frontotemporal dementia (IBMPFD). The patients were described as angry, apathetic, and suffering from anomia, which included frequent visual or aural hallucinations. Their memory was believed to be unaffected in most cases (Yeo and Yu 2016). VCP comprises of four structural domains which include highly conserved amino-terminal (N domain), two ATP-binding domains (D1 and D2), and a C-terminal region. The regions linking the N domain, D1 and D2 domains are named as N-D1 linker and D1–D2 linker (Xia et al. 2016) (Fig. 3.8a, b). The N-domain binds to a variety of substrates and facilitates the ubiquitin-proteasome degradation cascade, while D1 and D2 domains help in formation of the homohexameric form, and confer ATPase activity respectively (Fig. 3.8c). Further, the C-terminal region contains the nuclear localization signaling motif, and interacts with nuclear localization-specific proteins (Tang and Xia 2016). Under normal conditions, VCP mediates the degradation of misfolded proteins in the ER by interacting with various polyubiquitinylated misfolded proteins and arbitrates proteasomal degradation.

The popularly known interactors of VCP are p47, NPL4 (nuclear protein localization protein 4) (Fig. 3.8d), UBXD1 (UBX-domain containing protein 1), ubiquitin ligase gp78, ubiquitin fusion degradation 1L (UFD1), and TDP43 (Joshi et al. 2017; Guo and Qi 2017). The binding of VCP and its protein interactors prevents aggregation of misfolded proteins, a major pathological process in neurodegenerative diseases (Bayraktar et al. 2016). Other than ER proteins, VCP also associates with proteins present in mitochondria (Lizano et al. 2017; Tang and Xia 2016), by mediating E3 ligase Parkin-dependent mitophagy-associated degradation of misfolded protein with the help of its UFD1 and NP14 (Guo et al. 2016). Further VCP is known to play a crucial role in calcium homeostasis by regulating the

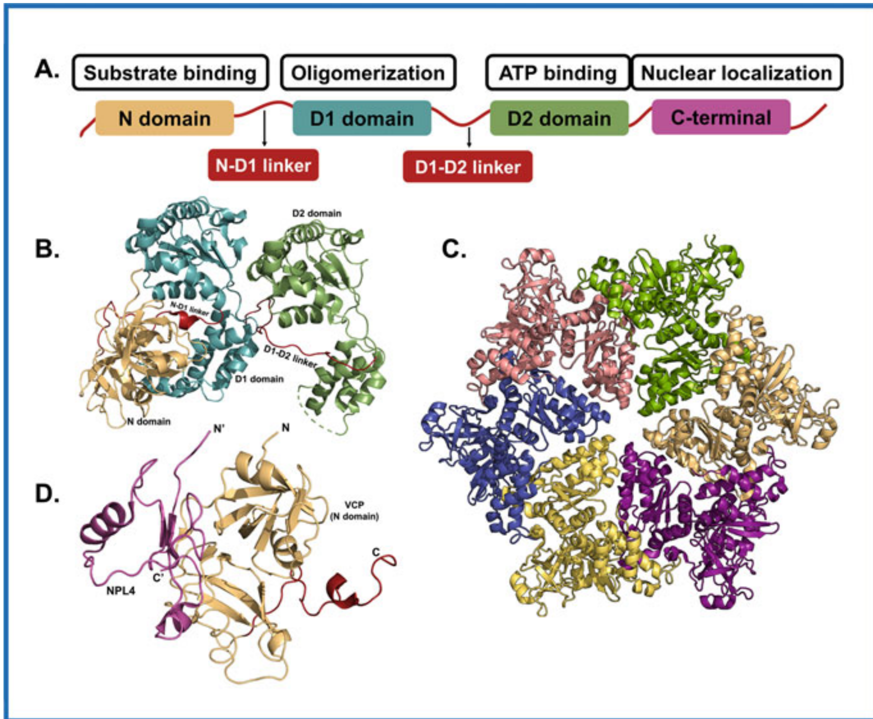

Fig. 3.8 Structural architecture of VCP protein. (**a**) Schematic representing the different domains of VCP protein. (**b**–**d**) represent monomeric, hexameric, and N-domain of VCP in bound state with one of its ligands—NPL4 (pink) (nuclear protein localization protein 4) respectively. The white, blue, green, and red in the monomeric state (**b**) represents the N, D1, D2, and linker regions respectively [PDB IDs: 3HU2, 1R7R, 2JPH]

mitochondrial calcium uptake proteins (MICU) (Patergnani et al. 2011). Through interactions with a number of ubiquitin adapters, VCP's major function is ubiquitin chain conjugation to its substrates and subsequent delivery of these substrates to the 26S proteasome for further degradation, like ER-associated protein degradation (ERAD) (Lan et al. 2017). IBMPFD-associated VCP mutations affect protein turnover via the ubiquitin proteasome system of the ER membrane, leading it to bind to other cofactors thus disrupting the UPS signaling cascade. These findings might be explained by the altered interactions between the proteins in the ERAD pathway, which impedes ubiquitinated proteins from being transported from the ER to the proteasome (Weihl et al. 2006). Furthermore, overexpression of two VCP mutants, R155H and A232E impairs autophagy by preventing ubiquitin-conjugated autophagosome maturation, resulting in an increase in autophagosome concentration, thus contributing to the FTD pathogenesis (Sun and Qiu 2020).

3.8.4 Chromatin-Modifying Protein 2B

The gene for chromatin-modifying protein 2B (*chmp2b*), also known as charged multivesicular body protein 2B (CHMP2B), is found on chromosome 3p11.2. CHMP2B is a member of a protein family that contributes to the endosomal sorting complex, which is essential for ESCRT-III (Endosomal Sorting Complexes required for Transport) type of transport. This multifaceted conjugate is found in neuronal cells throughout the brain, particularly in the regions of frontotemporal lobes, the hippocampus, and the cerebellum. During the creation of multivesicular bodies, it is considered that the transport-III complex contributes to the autophagic/late endosome pathway by degrading proteins of cell membrane receptor (Vandal et al. 2018).

As CHMP2B mutations impair the activity of the ESCRT-III complex, aberrant protein accumulates in huge cytoplasmic vacuoles. CHMP2B mutations are rather rare, accounting for less than 1% of all FTD hereditary mutations. The usual inception age is in the mid-50s, and the condition lasts for 10 years (Isaacs et al. 2011). The CHMP2B was first discovered in a Danish family with FTD linked to chromosome 3 (called FTD-3). Due to the splice site mutation, two transcripts are produced, each encoding basically two proteins with a faulty C-terminal: CHMP2Bintron5 and CHMP2Bdel10. In the first one, the intronic sequence for both exons 5 and 6 is preserved but only one valine is embedded rather than the full 36 amino acids programmed by exon 6; whereas, in the second secreted ambiguous 10 bp splice site from exon 6 is used. Later, a similar case was found in a Belgian family with familial FTD-3, with the mutation causing the protein to be truncated, losing 49 amino acids. The mutations related to amyotrophic lateral sclerosis (ALS) in CHMP2B, on the other hand, were missense mutations (Ranganathan et al. 2020).

3.8.5 Protein p62

The *sqstm1* gene on 5q35.3 gets translated to protein p62, binds to ubiquitin, and is important for NF-kB signaling, apoptotic, and autophagic pathway. p62 functions are carried out by protein–protein interactions, which are aided by a variety of domains (Miller et al. 2015). Its function as an autophagy receptor is mediated by two domains namely, the UBA domain which binds to ubiquitinated substrates, and its LC3-interacting domain that interacts with autophagosome membrane protein LC3 (LIR). Self-oligomerization is mediated by the PB1 domain of p62, which may be significant in the degradation of certain substrates via the ubiquitin-proteasome system (UPS) or macroautophagy (Rea et al. 2014). p62 also modulates signaling pathways that stimulate the transcription factors Nrf2 and nuclear factor kappa B (NF-κB), which are critical for brain health. In neurons, the Nrf2 pathway is the primary response to oxidative stress, and knocking it off makes cells more vulnerable to neurotoxic insults (Arai et al. 2003).

It is worth mentioning that p62 has been occupied in the positive control of NF-κB. As a result, p62 has a dual function in NF-κB regulation that is either p62 concentration-dependent or temporally regulated. p62 is a multifunctional protein

with several functional domains, mutations affecting these domains influence autophagy as well as cell signaling pathways including the Nrf2 and NF-κB, as well as mitophagy and aggregate/inclusion body formation (Itakura and Mizushima 2011). FTD-associated p62 polymorphisms may therefore contribute to the etiology by impaired toxic protein degradation, and a reduced capacity to develop an appropriate stress response, leaving cells more vulnerable to neurotoxic assault. The importance of p62 and modifiers like TBK1 in a healthy autophagy lysosome system is well understood, but further study is required to explore how mutations that impair mitochondrial turnover and altered NF-B signaling influence neuronal health and survival (Foster and Rea 2020).

3.8.6 Ubiquilin-2

Ubiquilin-2 is a single exon-encoded 66 kDa protein belonging to Ubiquilin family of proteins. The protein-coding gene *ubqln2* is positioned on Xp11.21 chromosome. UBQLN2 protein contains a ubiquitin-like domain (UBL) at the N-terminal that binds to the 26S proteasome, and a C-terminal ubiquitin-associated domain (UBA) detecting polyUb chains on ubiquitinated proteins (Walters et al. 2002) (Fig. 3.9a). It is cytosolic in nature and has been found in the brain, heart, spleen, liver, pancreas, and other organs. It is responsible for the protein homeostasis by guiding misfolded or absurd proteins to the proteasome. UBQLN2 is dormant normally, but becomes active when HSP70 interacts to proteins, exposing a UBQLN2-binding site. When UBQLN2 is activated, it binds to the 26S proteasome and forms a degradation-competent complex. It has also been associated with the protein breakdown in the endoplasmic reticulum (ER) (Xia et al. 2014). The UBA domain of UBQLN2 interacts with the ER membrane localized ubiquitin regulatory X domain-containing protein 8 (Ubxd8), assisting the transfer of substrate for ERAD to the cytoplasm. When activated under stress, UBQLN2 may potentially bind to Herp (homocysteine-induced endoplasmic reticulum membranes protein), which protects the cells by regulating Ca^{2+} homeostasis in ER and preserving the functioning of mitochondria (Fig. 3.9b) (Kim et al. 2008). Meanwhile UBL domain detects ubiquitinated proteins and leads them to the 26S proteasome. UBQLN2 is also able to induce autophagy through indirect interaction with autophagy-associated proteins such as LC3 (Croona 2020).

Mutated UBQLN2 has recently been discovered as genetic indicators for FTD. UBQLN2 mutations are known in 2% of rare autosomal FTD cases. The majority of the mutations have been found in a region of proline-rich PXX domain of UBQLN2; however, several changes outside of this region have recently been discovered. The PXX domain is often involved in PPIs. As a result, changes in this domain are likely to have a significant impact on UBQLN2 function (Hjerpe et al. 2016). Similar to the other proteins relevant to FTD, mutations in UBQLN2, cause aggregation and the formation of cytoplasmic inclusion bodies in the medulla spinalis and other brain tissues. These aggregated UBQLN2 acquires the potential of attaching them with other proteins like TAR, TDP-43, and FUS (Takada 2015). A detailed purview of

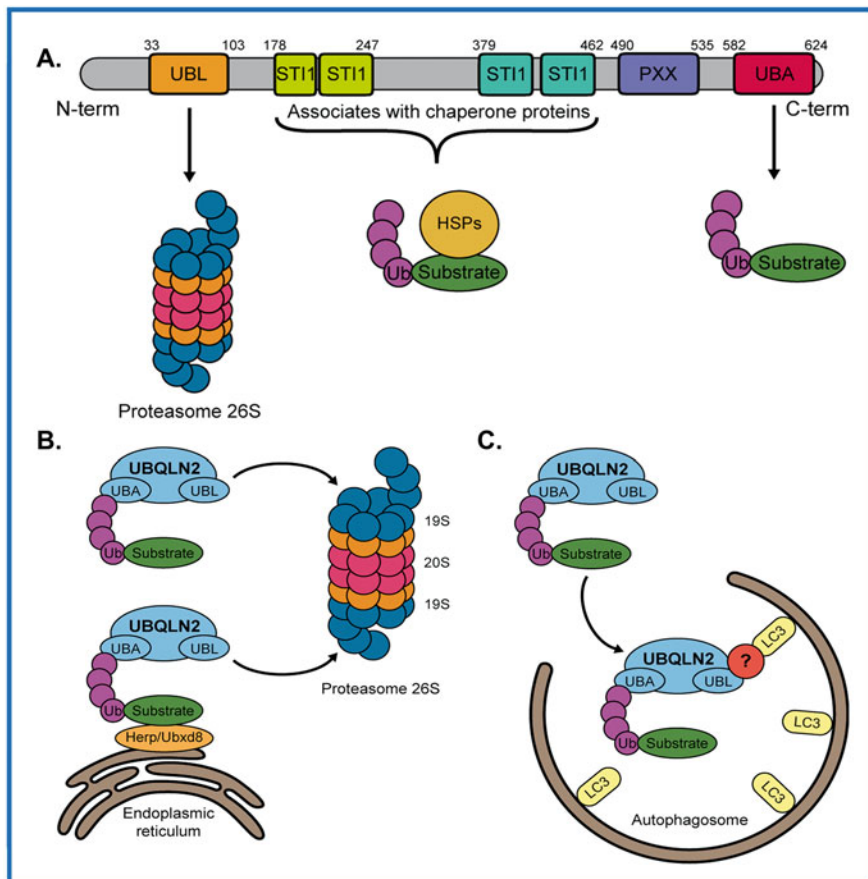

Fig. 3.9 Schematic representation of (**a**) UBQLN2 protein architecture, and (**b**, **c**) mechanism of interaction of UBQLN2 with proteasomal degradation and autophagy-associated protein. (Adapted from Renaud et al. 2019)

UBQLN2-TDP43 interactions has been recently reviewed by Renaud et al. (2019), and is explained below.

3.8.6.1 TDP43–UBQLN2 Interaction

TDP-43 intrinsically is a nuclear localized protein but in FTD, it is translocated to the cytoplasm and gets processed by caspases, producing phosphorylated and ubiquitinylated TDP-43 CTFs. Surprisingly, UBQLN2 mutations result in the accumulation of UBQLN2 and TDP-43 in the inclusion bodies called stress granules. Nevertheless, UBQLN2 and TDP-43 are often not found in the same inclusion body, and their degree of coexistence seems to be reliant on the position of the UBQLN2 mutation, with colocalization being prevalent in individuals with UBQLN2 mutations immediately upstream of the PXX domain (Daoud and Rouleau 2011).

Furthermore, when both UBQLN2 and carboxy terminal TDP-43 (218–414 aa) are overexpressed in cell culture models, they are copositioned in cytoplasmic aggregates. While the relevance of these results is unknown, they do imply that there may be a relationship between UBQLN2 and TDP-43 that merits further examination (Deng et al. 2011).

3.8.7 TREM2 Protein

TREM2 (Triggering Receptor Expressed on Myeloid cells 2) are membrane proteins present in receptor-mediated signaling complex with the TYRO protein (TYROBP) and are found on chromosome 6p21.2. This complex is involved in immune response activation, osteoclast and dendritic cell development, and microglial phagocytosis (N'Diaye et al. 2009). TREM2 is increased in activated microglia and plays a role in phagocytosis, survival, chemotaxis, and neuronal damage response. Homozygous TREM2 mutations cause Nasu-Hakola illness, a rare condition linked to early-onset of FTD-like dementia, while homozygous TREM2 mutations are linked to FTD-like disorders without bone involvement (Le Ber et al. 2014). TREM2 ectodomain is cleaved, releasing a soluble TREM2 (sTREM2) fragment into extracellular space in CSF. Although elevated CSF sTREM2 levels were first reported in neuroinflammatory illnesses like multiple sclerosis, the link between sTREM2 and other disease indicators has lately piqued attention in neurodegenerative disorders (Woollacott et al. 2018).

3.8.8 Tank-Binding Kinase 1 (TBK1)

Tank-binding kinase 1 (TBK1) is a serine threonine kinase with numerous activities, including the ubiquitinated cargo clearance as measure of the innate immune response and selective autophagy. It is found on chromosome 12q14.2 and is responsible for encoding a kinase to serve a wide range of functions. TBK1 substrates comprise p62 and optineurin, both of them are implicated in autophagy (Herhaus 2021). ALS and FTD both have been linked to loss-of-function mutations. Researchers have documented functional impacts of 25 TBK1 missense mutations discovered in ALS or FTD patients. They concentrate on faulty PPIs and substrate-definite abnormalities in innate immunity and autophagy processes. Patients having TBK1-linked FTD manifest clinically as bvFTD, albeit early loss of memory and bewilderment have been reported in association with the development of behavioral problems (Ye et al. 2019). The genetic relationship between TBK1 and neurological disorders is mostly centered on deleterious loss-of-function mutations (including splicing site or frameshift mutations) that are more prevalent in ALS/FTD patients, and leads to the lack of expression of one TBK1 gene (Gijselinck et al. 2015).

3.9 Batten Disease (Neuronal Ceroid Lipofuscinosis)

The neuronal ceroid lipofuscinoses (NCLs) are a cluster of recessive neurological illnesses defined by the buildup of indigestible ceroid lipofuscin in plentiful cell types, resulting in advanced neurodegeneration. The NCLs, also called as Batten disease, are responsible for severe clinical manifestation such as visual loss, motor and mental deterioration, epilepsy, and early mortality. Despite the fact that Batten disease affects people of all ages and ethnicities, it is known as the most frequent type of neurodegeneration among children (Radke et al. 2015).

Due to the general lysosomal buildup of ceroid lipofuscin, NCLs are usually referred to as lysosomal storage disorders. However, the specific activities of NCL-related genes and proteins, as well as the molecular and cellular mechanisms causing NCLs, are still unknown, spurring the development of a range of model systems to provide insights into the biological processes impacted by NCL-related gene alterations (Minnis et al. 2020). Early research on patient samples revealed that altered protein production was involved in NCLs. This is aligned with neural signaling being dependent on the controlled release of proteins and other chemicals, and any abnormality or mutation in the gene encoding for these proteins can lead to the impaired secretion and ultimately cause neurodegeneration. Till date, occurrence of 13 distinct types of neuronal ceroid lipofuscinosis (knowns as CLNs) caused by mutation in the lysosomal and ER-associated protein (CLN1–CLN8 and CLN10–CLN14) have been reported, and are summarized by Butz et al. (2020). Based on the function of each associated protein, CLNs can be clustered into five groups, NCL associated (1) lysosomal enzyme deficiencies, (2) soluble lysosomal protein, (3) lysosomal membrane protein, (4) ER-localized protein, and (5) nonlysosomal proteins. Although the phenotypic effects of all CLN-associated proteins have been identified, the exact binding substrate/proteins are only known for some of the CLN diseases. Various CLN-associated proteins, their known interactors, and their effects/functions have been summarized in Table 3.5.

3.10 Conclusion

Neurodegeneration is a compounded class of disorders and by the end of the 2050, estimated around 115 million patients will be affected by at least some type of neuronal disease. Discrepancy in folding, aggregation, and protein–protein interaction (PPI) involving an array of proteins like amyloid-β, tau, α-synuclein, PrP, CDK5, cathepsin, and many more leads to this protienopathy. An arena of both experimental and computational research is ongoing at a rapid pace to decipher the molecular basis, subvert, and cure this pressing issue. This chapter systematically described the details of all majorly reported neurodegenerative diseases and behavioral aspects of the associated causal proteins in triggering the pathophysiology. The data undoubtedly represent the indispensable role of the hampered protein–protein interaction network, and a conclusive mechanistic idea about the molecular interactions enabling us to design, generate, and repurpose molecules/drugs

Table 3.5 Summary of CLN disease-associated proteins and effect of associated protein-protein interactions

CLN type	Associated protein	Type of protein	Known substrates	Effect of mutation in diseased state	References
Lysosomal enzyme deficiencies					
CLN1	Palmitoyl-protein thio-esterase1 (PPT1)	Thio-esterase	Cysteine-sting protein alpha (CSPα), NMDA receptors, V-type ATPases, cytoskeletal proteins, voltage-gated calcium ions channels	PPT1 mislocalization; hyperpalmitoylation of the NMDAR; elevated lysosomal pH in cells; morphological effects	Koster et al. (2019), Appu et al. (2019)
CLN2	Tripeptidyl-peptidase 1 (TTP1)	Serine protease	Peptide hormones, mitochondrial ATP synthase subunit C	NA	Pal et al. (2009), Schulz et al. (2018)
CLN10	Cathepsin D (CTSD)	Aspartic protease	Huntingtin, α-synuclein, amyloid precursor protein	Impairs biogenesis and synaptic transmission in GABAergic neurons leading to hyperactivity and seizures	Vidoni et al. (2016), Tayebi et al. (2020)
CLN13	Cathepsin F (CTSF)	Cysteine protease	Lysosomal integral membrane protein type-2 (LIMP-2)	Prevented LIMP-2 processing leading to irregularities in lysosomal targeted protein transport	Peters et al. (2015)
Soluble lysosomal proteins					
CLN5	CLN5	Glycoside hydrolase	Rab5, Rab7, PPT1, CerS, Vimentin, TPP1, CLN3, CLN6, CLN8, CALR3, H2AFZ/H3F3A/H1H4H/ H2A type 2-C FBXo6, OBP2A, LIPH	MPR and sortilin lysosomal degradation resulting in defective recycling machinery of lysosomal proteins	Basak et al. (2021)
CLN11	Progranulin	Glyco-protein	Prosaposin	Dysregulation of lysosomal lipid homeostasis	Du et al. (2022)
Lysosomal membrane proteins					
CLN3	CLN3	Transporter or ion channel	Kv4.2/KChIP3, Rab7A	Multiple lysosomal enzymes deficiency; ionic imbalance, and autophagy-lysosomal pathway flux leading to lysosomal dysregulated	Mole et al. (2019), Seifert et al. (2020), Yasa et al. (2020)

(continued)

Table 3.5 (continued)

CLN type	Associated protein	Type of protein	Known substrates	Effect of mutation in diseased state	References
CLN7	MFSD8	Lysosomal membrane protein	NA	Accumulation of lysosomal storage material in cranial region, neuroinflammation, neurodegeneration and altered autophagy	(Chen et al. 2022) (Steenhuis et al. 2012)
CLN12	ATP13A2	P-type ATPase	Cations, heavy metals, and lipids	Altered lysosomal morphology; accumulation of storage material; α-synuclein aggregation; impaired mitochondrial function	Specchio et al. (2021), Marcos et al. (2019)
ER-localized proteins					
CLN6	CLN6	NA	NA	Disruption in lysosomal homeostasis	Butz et al. (2020)
CLN8	CLN8	TRAM-Lag1p-CLN8 (TLC)-domain-containing protein	CTSD, CTSF, TPP1, PPT1	Disruption in lysosomal homeostasis by depletion of a soluble lysosome proteins	di Ronza et al. (2018)
Nonlysosomal proteins					
CLN4	Cysteine-string protein alpha (CSPα)	Chaperone	PPT1	Misfolding and instability of various synaptic proteins; disassembly of SNARE complex, required for translocation of synaptic vesicles	Nieto-González et al. (2019), Naseri et al. (2021)
CLN14	KCTD7	Potassium channel tetramerization domain-containing protein	Cullin-3	Defect in autophagy and/or mitophagy	Wang et al. (2022), Metz et al. (2018)

targeting the underlying protein–protein interaction network. Despite rigorous research to find PPI interaction blockers of neuropathy, no single molecule/drug/ combination has been approved by the FDA till date. In this context, it is worth mentioning that a combinatorial reappraisal of two FDA-approved drugs, cromolyn and ibuprofen has proven to be useful in suppressing αβ accumulation, inflammation, and subsequent neuronal death. In adjunct to discovering PPI inhibitors, it is vital to identify relevant biomarkers for the disease diagnosis and prognosis of differential neuropathic conditions. Extensive interdisciplinary and profound investigation are quintessential to develop all-inclusive prevention and cure strategies for each type of these neurodegenerative disorders.

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Protein–Protein Interactions in Immune Disorders and Inflammation

4

4.1 Overview of Inflammation

Inflammation is a biologically conserved event in the complex immunological response of the human body against any foreign stress, such as infection (pathogens and irritants) or injury (injured cells). Inflammation acts as the protector, eradicates the initial cause, and also induces the process of tissue regeneration, which is considered as the standard response machinery of innate immunity (Abbas et al. 2019). Redness, heat, swelling, pain, and loss of function are the five traditional insignia of inflammation, though it is not limited to these responses only. The representative Fig. 4.1 below describes the initiation of an inflammatory reaction involving various biochemical pathways and protein–protein interactions. Inflammation is a relatively short-lived response that helps the immune system to eradicate the pathogen or heal a lesion. However, persistent and allergic inflammations are prevalent and may be dangerous (Hawiger and Zienkiewicz 2019).

An inflammatory response is arbitrated to ascertain the danger created by the infectious cells. For example, a microbial infection can cause inflammation, which stimulates specific antimicrobial molecules within the cell; while wound healing responses can be produced after a skin injury. Although differential cellular responses arise under variable stress conditions, the inflammatory cell signaling is primarily controlled by the master regulator NF-κB (Koh and DiPietro 2011). Inflammation can be broadly categorized as acute and chronic inflammation. Acute inflammation starts shortly after an injury and lasts for a few days, whereas the chronic inflammation lasts for months or in some cases, even for years. In chronic inflammation, macrophages, lymphocytes, and plasma cells predominate, while neutrophils take precedence in the case of acute inflammation (Hawiger and Zienkiewicz 2019).

In the process of inflammation, pattern recognition receptor proteins (PRRs) comprising of toll-like receptors (TLRs), play a significant role in sensing the external stimulus. TLRs are the group of membrane-bound proteins in which

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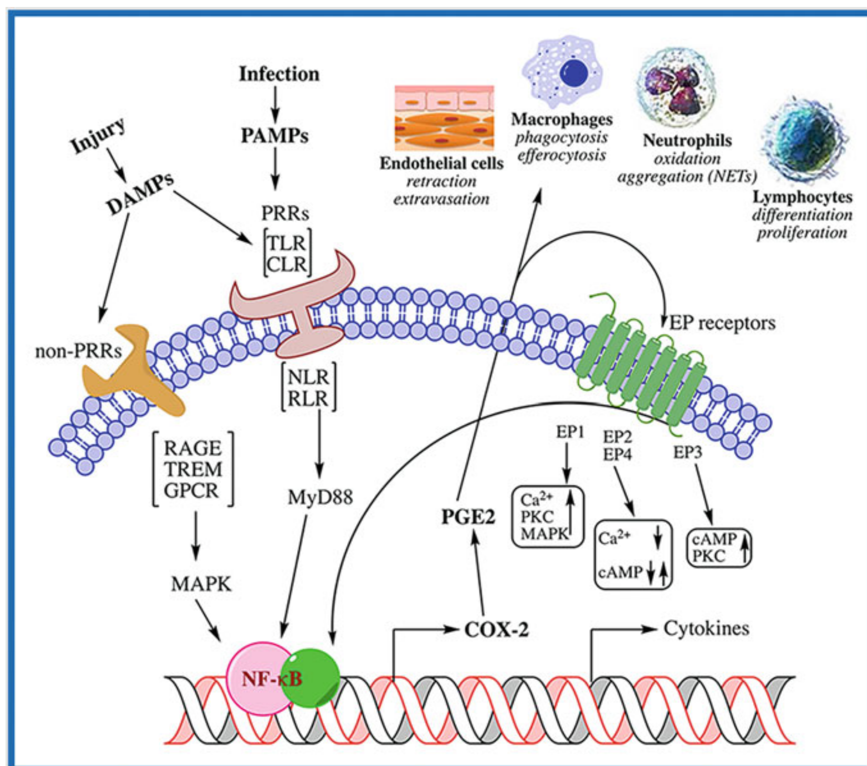

Fig. 4.1 Summary of protein–protein interactions and biochemical pathways leading to inflammation. (Adapted from Maddipati 2020)

dimerization is triggered to sense the distinctive molecular patterns. In case of pathogenic stress, it recognizes the Pathogen-Associated Molecular Patterns (PAMPs); whereas, Damage-Associated Molecular Patterns (DAMPs) are sensed in the case of tissue injury (Tang et al. 2012). Upon sensing the PAMPs and/or DAMPs, immune cells stimulate the cascade of chemical changes that are initiated with the production of small messenger proteins called cytokines, which converses the information of external stimulus to the other immune cells through autocrine, paracrine or endocrine signaling to eliminate the infection or heal the lesion. Table 4.1 provides a summary of the key cytokines implicated in the process of inflammation (Kany et al. 2019).

There are numerous proteins involved in the process of inflammation; nuclear factor-kappaB (NF-κB) is the most important one among them as mentioned earlier. NF-κB is a transcription factor that regulates the group of proteins that are crucial for governing the genes responsible for inflammation and immunity. It triggers the genes in the cells of innate immunity that are present in macrophages, dendritic cells, and neutrophils. Apart from this, NF-κB is also observed to be involved in the adaptive immune response by governing T cell activation and differentiation (Liu

Table 4.1 List of some essential cytokines that play major role in inflammatory responses

Cytokines	Description	References
IL-1	– Proinflammatory cytokine produced by stimulated macrophages, monocytes, and keratinocytes. – Thymocyte proliferation is stimulated by IL-1 alpha, as is B cell proliferation and maturation.	Gabay et al. (2010)
IL-6	– Generated by activated T cells, macrophages, endothelial cells, fibroblasts, and osteoblasts. – IL-6 stimulates cells by soluble IL-6R serpentine receptor. – Along with B cell differentiation and immunoglobulin synthesis, it also promotes hybridoma proliferation.	Tanaka et al. (2014)
IL-8	– Endothelial cells and monocytes produce CXCL8. – IL-8 attracts neutrophils, T cells, and basophils through the CXCR1 and CXCR2 receptors.	Ghasemi et al. (2011)
IL-10	– Initially referred as CSIF (cytokine synthesis inhibiting factor). T cells, mast cells, B cells, and macrophages all secrete IL-10. – TNF-α, IL-1, IL-2, IL-3, and IL-8 are among the proinflammatory cytokines that IL-10 inhibits.	Boonpiyathad et al. (2019)
IL-33	– Endothelial venules generate IL-33 in tonsils, Peyer's patches, and mesenteric lymph nodes. – IL-33 inhibits T helper 2 (Th2) cell cytokine production by binding to the receptors of ST2 and toll-interleukin 1 (IL-1).	Di Salvo et al. (2018)
TNF-α	– Generated by the macrophages, T and B lymphocytes, stimulated monocytes, and fibroblasts. – TNF-α stimulates the transcription factor NF-kappa B, induces cell death in several cancerous cell, and promotes cell growth. – TNF-α involved in inflammation, septic shock, autoimmune diseases, and in the case of chronic RA.	Zelová and Hošek (2013)

et al. 2017). In physiological conditions, NF-κB stays inactive in the cytoplasm due to its association with inhibitor kappa B alpha (IκBα). In response to an external stress or inflammatory cytokine-like tumor necrosis factor alpha (TNF-α), IκBα is cleaved and destroyed by proteolytic activity, in turn releasing the NF-κB that migrates to the nucleus and promotes the transcription of genes that are responsible for the initiation and maintenance of inflammation (Mussbacher et al. 2019). A bunch of other proteins associated with major inflammatory cell lines like neutrophils, monocytes, and macrophages orchestrate diverse inflammatory disorders on interacting with each other in unregulated manner (Chaplin 2010).

An immune system performing correctly will only be activated upon the introduction of an actual threat, and upon removal of the stimulus, the inflammatory response would be terminated. Conversely, immune system can also induce inflammation against nonharmful elements and in some cases, it fails to differentiate between the self and the foreign elements and generates response against self, which is termed as autoimmune inflammation. If this stimulus is persistent, it may lead to symptoms of many chronic pathophysiological conditions like cardiovascular

disorders, autoimmune diseases, and neurodegenerative disorders like Alzheimer's. For instance, abnormality in the NF-κB signaling is established to be the signature symptoms of immune disorders that include rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis, type I diabetes, and asthma. Owing to the fact that NF-κB is the principal controller of inflammation involved in the activation of numerous proteins governing the phenomenon of innate immunity, aberration in the activity of these proteins may lead to the perturbed immune response (Liu et al. 2017). The NF-κB, associated proteins, and its multifaceted network of molecular interactions exhibit a pivotal function in triggering immune responses and are distinctively involved in the onset and progression of various immune diseases. Targeting the NF-κB and associated protein–protein interaction interfaces can be a potent therapeutic approach, regulating or reversing the chronic state of the inflammation (Hsu and Katelaris 2009).

4.2 Types of Immune Disorders

In general, any dysfunction in the immune system is implied as immune disorders. Immune disorders can be classified on the basis of any immune condition being congenital or acquired; and also, it can be categorized based upon the constituents of immune system that are much affected considering the fact that immune system can be underactive or overactive. There are various immune disorders and these can be broadly classified into three major categories that include; hypersensitivity disorders, immunodeficiency disorders, and autoimmune disorders (Zeher and Szegedi 2007).

4.2.1 Hypersensitivity Disorders

In the physiological conditions, hypersensitivity can be defined as the unwanted response of the immune system. Hypersensitivity reactions are immune responses to harmless antigens that result in symptomatic reactions when reexposed. If this occurs often enough, they may lead to hypersensitivity disorders. Hypersensitivity is a term used to describe a condition of increased responsiveness to antigen and the associated disorders can be categorized into four classes as type I–IV (Gell and Coomb's classification) hypersensitivity as described in Table 4.2 (Uzzaman and Cho 2012).

4.2.2 Immunodeficiency Disorders

The compromised activity of the immune system leads to the reduction in its capacity to protect the body against infectious pathogens as it fails to neutralize the invading foreign stresses including bacteria, virus, and fungus inducing the development and reoccurrence of a severe infection that stays for longer period, thus leading to disorders known as immunodeficiency disorders (Table 4.3). These

Table 4.2 Gell and Coombs classification of hypersensitivity

Type	Name	Immune cells involved	Examples of diseases	References
Type-I hypersensitivity	Anaphylactic	IgE	– Hay fever – Asthma – Anaphylactic shock	Abbas et al. (2021)
Type-II hypersensitivity	Cytotoxic	IgM, IgG, and complement system	– Transfusion reaction – Hemolytic diseases of newborn (HDN)	Dispenza (2019)
Type-III hypersensitivity	Complex-mediated	IgG	– Alveolitis – Serum sickness	Usman and Annamaraju (2021)
Type-IV hypersensitivity	Delayed-type hypersensitivity (DTH)	Sensitized CD4+ T lymphocyte	– Mantoux reaction – Contact hypersensitivity	Marwa and Kondamudi (2021)

disorders can also be a resultant of prolonged use of any drug or due to life threatening diseases like cancer. In some cases, these disorders are established to be inherited also. Broadly, the immunodeficiency disorders can either be primary as observed in Burton's diseases or secondary, like the one that is induced by the HIV infections (Justiz Vaillant and Qurie 2018). Primary deficiency is caused by starvation, which inhibits cell-mediated immunity and phagocytosis. Though the ingestion of foreign organisms is not affected, but the ability of phagocytic cells to eliminate intracellular organisms is impaired (McCusker et al. 2018). On contrary, secondary immunodeficiency is induced by the drugs like steroids, cyclophosphamide, azathioprine, mycophenolate, methotrexate, leflunomide, ciclosporin, tacrolimus, rapamycin, etc. Moreover, viruses are also reported to induce secondary immunodeficiency; in the case of HIV infection, when virus impaired the activity of CD4+ T cells and suppresses cellular immune responses, it facilitates the opportunistic infections that are deleterious to the human health (Zicari et al. 2019).

4.2.3 Autoimmune Disorders

Autoimmunity can be defined as a physiological aberration where the immune system starts acting abnormal and targeting its own cells. Autoimmune disorders have a complicated origin, with active participation of genetic, hormonal, and environmental variables (Chen et al. 2016). In autoimmune disorders, an aberrant response to self-antigen identification at the cellular level manifests itself in a broad range of clinical manifestations, wherein most of them are severe and have a considerable effect on the quality of life (Ngo et al. 2014). Disease categorization in the case of autoimmune disease is very ambiguous, as there is the lack of thorough

Table 4.3 Type of immunodeficiency, its cause, and major disorders associated

Types of ID	Cause	Example of disorders	References
Primary	B cell deficiency	Bruton disease	Hernandez-Trujillo et al. (2014)
	IgA deficiency	Recurrent sinus and lung infections	Moschese et al. (2019)
	T cell deficiency	DiGeorge syndrome	Lambert et al. (2018)
		Chronic mucocutaneous candidiasis	Carey et al. (2019)
	Deficiency of both T-cell and B-cell	Severe combined immunodeficiency disease	Chinn and Shearer (2015)
		Wiskott-Aldrich syndrome	Byrne et al. (2018)
		Bare leukocyte syndrome	Aluri et al. (2018)
	Complement system deficiency	Hereditary angioedema	Kirschfink and Mollnes (2003)
		SLE	Dosanjh (2015)
	Phagocyte deficiency	Chronic granulomatous disease (CGD)	Zhou et al. (2018)
		Leukocyte adhesion deficiency syndrome	De Rose et al. (2018)
Secondary	Use of drugs (steroids)	Multiple disorders induced by B cell and T cell deficiency	Ocon et al. (2017)
	Nutrient deficiencies	Antibody and cytokine production is impaired	Rehman et al. (2017)
	Obesity	Compromised NK cell-promoted disorders	Justiz Vaillant and Qurie (2018)
	Acquired immune deficiency syndrome	Impaired T cell-induced disorders	Tchatchouang et al. (2019)

knowledge of the underlying processes. Broadly, based on the affected organs, it can be classified in two types: those in which autoimmunity is expressed only in certain organs of the body ("organ-specific"), and those in which multiple tissues of the body are harmed are known as systemic autoimmune disorders. Table 4.4 below describes the categorization of the major diseases under each category (Janeway et al. 2001).

4.3 Multiple Sclerosis

Multiple sclerosis (MS) is an ailment of CNS that can have a deleterious impact on both the brain and the spinal cord. MS has a pathobiology that involves autoimmunity damaging the myelin sheath of nerves, thus disrupting the communication between the brain and other body components (Fig. 4.2) (Islam et al. 2019). MS

Table 4.4 Types of autoimmune disorder with pathological manifestations

Types of autoimmune disorder	Diseases	Pathological characteristics	References
Organ-specific autoimmune disorder	Multiple sclerosis	– Cognitive impairment – Optic neuritis – Cerebellar syndromes	Sand (2015)
	Type 1 diabetes	– Hyperglycemia – Polyuria – Polydipsia	Rashtak and Pittelkow (2008)
	Inflammatory bowel disease – Crohn disease – Ulcerative colitis	– Fistulas – Erythema nodosum – Pyoderma gangrenosum – Ulcer	Ferré et al. (2018)
	Ankylosing spondylitis	– Acro-iliac joint tenderness – Peripheral arthritis	Golder and Schachna (2013)
Systemic autoimmune disorder	Systemic lupus erythematosus (SLE)	– Lupus nephritis – Photosensitivity – Serositis – Malar rash	Tsokos (2011)
	Sjogren syndrome	– Xerophthalmia – Xerostomia	Rashtak and Pittelkow (2008)
	Sarcoidosis	– Dermatologic aberration – Cardiac manifestation – Neurological disorders	Jain et al. (2020)
	Rheumatoid arthritis	– Synovial inflammation – Amyloidosis – Systemic vasculitis	Rashtak and Pittelkow (2008)
	Celiac disease	– Diarrhea – Malabsorption of food	Lebwohl et al. (2018)

has a very unpredictable and diverse course, in most patients, the disease begins with reversible neurological impairments, which are frequently followed by gradual neurological deterioration over time (Goldenberg 2012). MS is a complicated

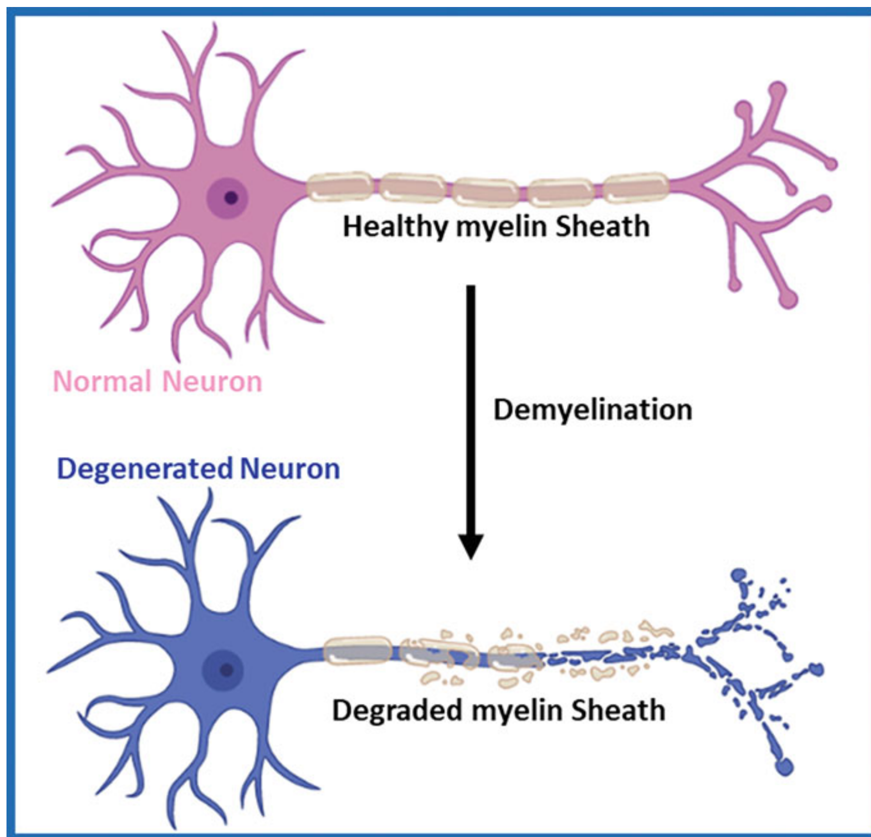

Fig. 4.2 Demyelination of myelin sheath in MS diseases

ailment in which a number of genes, along with a numerous precise environmental factor such as vitamin D or ultraviolet-B light (UV-B) exposure, Epstein–Barr virus (EBV) infection, obesity, and smoking, all contribute to disease vulnerability (Palacios et al. 2011). The occurrence of MS is typical but not restricted to the people amid the ages of 20 to 45, with chances of occurrence in infancy and late middle life as well (Dobson and Giovannoni 2019). Multiple sclerosis has a highly varied natural history, with symptoms that may come, vanish, recur, or worsen along with the time. In spite of this diversity, the disease can be broadly categorized into relapsing and progressing which are further subdivided into RRMS (Relapsing remitting multiple sclerosis) and CIS (Clinically Isolated Syndrome), and PPMS (Primary Progressive Multiple Sclerosis) and SPMS (Secondary Progressive Multiple Sclerosis) respectively (Leary et al. 2005).

Multiple sclerosis is assumed to be caused by diverse factors that include environmental and genetic mutations in dozens of genes, though, the exact cause remains unclear. The strongest genetic risk factors for this ailment are alteration in

the HLA-DRB1 gene, HLA classes I and II, T-cell receptor, CTLA4, ICAM1, and SH2D2A (Sadovnick 2012). Alteration in the IL7R gene, as well as environmental factors like Epstein-Barr virus exposure, low vitamin D levels, and smoking, have all been linked to an increased chance of developing multiple sclerosis. Vitamin D also has a critical participation in the MS causative chain. This includes evidence of a gene–environment interaction between vitamin D and the major MS-linked HLA-DRB1*1501 allele, and an indication that vitamin D levels in MS patients are substantially lower than in controls (Handunnetthi et al. 2010). As, HLA-DRB1 and IL-7R genes are pivotally involved in the immune system, any alterations in either of the genes could be linked to the autoimmune response that destroys the myelin sheath and nerve cells, resulting in multiple sclerosis signs and symptoms (Brynedal et al. 2007).

4.3.1 Myelin Basic Protein (MBP) and Peptidylarginine Deiminases (PADs)

Myelin basic protein (MBP) is one of the significant proteins of myelin sheath, synthesized by oligodendroglial cells and is responsible for maintaining its stability. MBP is believed to interact with the negatively charged lipids at the surface of cytoplasm, thereby, inducing the regulatory surface adhesion for the myelin sheath condensation (Hu et al. 2004). MBP proteins go through post-translational modifications which, govern their structural and functional aspects, simultaneously regulating their affinity to any ligand. Citrullination, a PTM occurs in MBP protein catalyzed by the enzyme peptidylarginine deiminases (PADs, EC 3.5.3.15) that induces the conversion of arginine to citrulline upon loss of a positive charge and release of ammonia. Citrullination in its vital state is a crucial element for regulating the gene expression, cell death, and flexibility of CNS. However, unusual citrullination leads to a new paradigm of cellular physiology, which, in turn, is responsible for the commencement and development of multiple sclerosis (Yang et al. 2016). Citrullination of MBP not only distresses the packaging of myelin sheath, but also induces T cells to work against the brain tissues (Moscarello et al. 2007). The T cell response to citrullinated MBP is observed to be enhanced and is reported to induce the MS development by breaching the Blood–Brain Barrier (BBB) and aiding in infiltration of the immune cells into the CNS. Moreover, MBP-reactive T cell-induced proinflammatory cytokines activate the immune cells such as macrophages and microglia to facilitate the disruption of axon. Through the process of neurodegeneration, matrix metalloproteinase-arbitrated exposure of immunodominant MBP epitopes triggers the MBP-specific T cell clone development, which assists the shift from acute to chronic neuropathic pain (Liu et al. 2012).

Inhibition of the MBP/PAD interface promotes the reversal of inflammatory condition, and can be a probable therapeutic approach for the treatment of conditions like MS. Researchers have studied the effect of mitotic inhibitor paclitaxel (toxol) on PAD and are reported to trigger the remyelination of previously damaged axons (Mastronardi et al. 2007). Similarly, 2-chloroacetamidine (2CA) can also be a

promising inhibitor of MBP/PAD interface. As hypercitrullination of MBP is one of the hallmarks of MS, 2CA in mouse model is described to act against the process of citrullination promoting the reduction of citrullinated proteins from the region of brain and spinal cord as well. Further, this promotes the clamping down of T cell autoreactivity against brain cells, and in turn facilitates the clearance of infiltrates from the CNS and remyelination of myelin sheath (Moscarello et al. 2013). These findings indicate the beneficial aspects of PAD inhibitors in the process of MS treatment and promote the development of highly efficient PAD inhibitors.

4.3.2 Fatty Acid-Binding Proteins (FABPs)

FABPs are the group of small proteins, having the molecular weight in the range of 14–15 kDa that act as the lipid chaperone to control the transportation of long chain fatty acid. All the 12 species of FABPs are tissue-specific proteins, among them FABP5/7 are expressed in the brain cells (Veerkamp et al. 1991). FABP5 is reported to be involved in the regulation of N-acylethanolamine (NAE) pathways. NAE are the vital signaling lipids that are responsible for the activation of cannabinoid receptors (CB), oleoylethanolamide (OEA), and palmitoylethanolamide (PEA) receptors, which arbitrate the signaling cascade through nuclear peroxisome proliferator-activated receptor (PPAR) (Cheng et al. 2021). In due course of process, FABPs facilitate the transportation of extracellular NAE from the cytoplasmic region to the fatty acid-amide hydrolase (FAAH) enzyme that hydrolyzes the lipid NAE, and subsequently activates the aforementioned receptors, which in turn induces the signaling cascade responsible for the initiation and development of MS (Shinoda et al. 2020). Consequently, it is evident that the pharmacological inhibition of FABP can be a potential strategy for the treatment of MS. Many small-molecule inhibitors have been reported to bind to the lipid binding site of the FABP 5/7 and inhibit its activity of lipid transportation. For instance, FABP ligand 6 (MF6) is demonstrated to have both the immune inhibition and oligodendrocyte protection (Cheng et al. 2021). In a recent computational study, Zhou et al. reported the compounds R, R-STK-15, and R,S-STK-15 that bind with FABP5 with high affinity and lead to its functional inhibition (Zhou et al. 2019).

4.3.3 Proteolipid Protein (PLP)

Proteolipid protein is an integral membrane lipid protein that is hydrophobic in nature and accounts for 50% of the proteins associated with adult CNS myelin. The PLP gene produces a polypeptide of 276-amino acid that is organized in five membrane-spanning domains having hydrophobic characteristics that bind to the lipid bilayer of myelin and aid in the creation of dense myelin comprised of multiple lamellar regions. PLP has a molecular weight ~ 30 kDa, and the PLP codon sequences are substantially conserved in humans, rats, and mice (Inoue 2005). Many PLP encephalitogenic peptides have been shown to cause experimental

autoimmune encephalomyelitis (EAE), based on which, it has been postulated that PLP might be crucially involved in MS. When EAE is induced in SJL/J mice, the main response of T cell is focused against PLP139–151, and epitope dispersion to PLP178–191 occurs during disease relapse (Vanderlugt and Miller 2002). Th1-induced immune response is developed upon injecting PLP peptides in mice and rats. This response leads to the infiltration of T cell in the region of spinal cord and initiates paralytic responses of varying degrees. The PLP peptides PLP104–117, PLP142–153, and PLP190–209 are the epitopes of immunological significance that interact with HLA-DR2 protein associated with pathogenesis of MS in humans, and are encephalitogenic peptides in animals (Greer et al. 1997).

4.4 Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a disorder of immune system distinguished by the generation of antibodies against the self-antigens and is mostly observed in the circulatory system. Upon manifestation of SLE, both the innate and adaptive immune system is in a compromised state that promotes the targeting of self-antigen. Aggregation of antigen-antibody complex takes place in the vital organs including liver, heart, kidney, and infrequently in the joints as well. In majority of the cases, SLE progresses toward its chronic condition described as lupus nephritis (LN) (Nikpour et al. 2014). Considering the involvement of multiple factors (genetic and/or environmental) in the progression of SLE, the etiology of SLE is tough to understand. The co-occurrence of SLE is reported in the identical twins that direct toward the robust genetic contribution in SLE, yet the exact inheritance pattern is not fully explained yet (Deng and Tsao 2014). Moreover, environmental factors and use of drugs like procainamide and hydralazine are established to be SLE inducing either by causing the demethylation of DNA or/and by modifying the self-antigen (Solhjoo et al. 2021). Pathogenesis of SLE is a multifaceted phenomenon that is periodically evolving.

Activation of the immune system against self-antigen due to infection and environmental factors results in the instigation of T cell and B cell-specific immune responses. Apart from these, it also facilitates the modulation of immunological factor including release of cytokines, activation of complement system, and production of antibody (Justiz Vaillant and Qurie 2018). An abnormal tolerance for autoantigens and upregulated neutrophil-mediated apoptosis are the key etiological factors of SLE. Antigen-presenting cells (APCs) like dendritic cells modulate the autoantigens and present them on to the surface, which is sensed by the T-cells and is followed by activation of the B cell for generating functional antibodies. These antigen-antibody complexes are deposited in the glomerular region of the kidney, and promote kidney damaging (Qi et al. 2018). In addition to this, proteins like heat shock protein (HSP), chemokines, calreticulin (CRT) form a protein–protein interactome with their cognate protein partners including heat shock factor (HSF), GPCRs, and TRIM2 protein respectively, and facilitate the activation of undesirable immune response that leads to the pathophysiological manifestation of SLE.

4.4.1 Heat Shock Protein (HSP)

HSP is a well-known stress-related protein involved in cytoprotection of oligomeric proteins tending in its folding, assembling, and transmigration in the intracellular region. HSPs are universally expressed proteins that are classified based upon their molecular weight (for instance 90 kDa; HSP90) (Shukla and Pitha 2012). HSP proteins are the molecular chaperones that govern the process of protein refolding, and prevent the proteins from degrading and creating a subcellular distress. Under physiological conditions, HSPs are controlled through its interaction with a transcription factor called heat shock factor (HSF), whereas, in stressful conditions, the protein–protein complex of HSP-HSF dissociates, and HSP migrates to nuclear region and upsurges the expression of HSPs (Pockley 2002).

According to the previous studies, antibodies against HSPs, specifically HSP90, are reported to be overexpressed in the plasma of patient with SLE manifestation (Mackern-Oberti et al. 2015; Shukla and Pitha 2012). In the stress conditions, HSP90 translocated to the nucleus and may interact with the dsDNA. At the time of necrosis, this DNA-bound HSP90 is discharged in the extracellular region initiating the generation of IFN-α, which further induces the proinflammatory responses (Ripley et al. 2001). Reduction of HSP90 in the extracellular region due to the immune activity leads to the downregulation of IFN-α production. On the contrary, in SLE patients, IFN-α production is unregulated and overexpressed that promotes the onset of lupus (Saito et al. 2015). Furthermore, HSP90 is primarily essential for the activation of client proteins that are obligatorily involved in the numerous signaling pathways regulating the cellular functioning. Considering this, extracellular HSP90 is reported to interact with protein kinase B (AKT) and facilitating its association with GSK3β. AKT/GSK3β complex is shown to activate the proliferation of lymphocytes, thus inducing the symptoms of SLE. These findings indicate that extracellular HSP90 is crucial for the development and progression of SLE, and inhibiting HSP90 could be a potential therapeutic strategy (Hong et al. 2020).

In recent studies, HSP90 has been explored as a target of inhibitor molecules for the treatment of SLE. In line with this, STA9090/ganetespib, a resorcinolic triazolone inhibitor has been studied against the autoimmune mice and it showed equal efficacy in comparison to the standard immunosuppressive cyclophosphamide. Moreover, the synergic effect of STA9090/ganetespib and cyclophosphamide in a periodic manner has shown to be having maximum disease control efficiency rather than the individual compounds (Proia et al. 2011). Hence, inhibitors like STA9090/ganetespib and 17-AAG restrict the HSP90 interaction with AKT, thus the formation of AKT/GSK3β protein complex and allied signaling cascade gets blocked eventually, thus providing the first-line evidence of HSP90 inhibitors as therapeutic agents for SLE (Daneri Becerra and Galigniana 2016).

4.4.2 Chemokines

Chemokines are the small chemotactic proteins of cytokine family having molecular weight in the range of 8–15 kDa. Chemokines function by interacting with the GPCRs expressed majorly over the surface of the leukocytes (Poluri 2014). Upon association with the receptors, chemokines arbitrate the signal transduction via various kinase proteins present inside the cells. Chemokines are classified into C, CC, CXC, CX3C depending upon the positioning of the cysteine residues in the structure (Tripathi and Poluri 2020). Being an integral part of the immune response machinery, chemokines are very crucial in the development and advancement of the autoimmune disorders like SLE as described below.

CXCL13, a member of CXC family is observed to be highly expressed in the case of SLE. Cells like B-Lymphocytes and CD4+ T cells express the receptor CXCR5 that facilitates the binding of CXCL13 and induces immune response. Along with the chemoattractant activity, CXCL13 is significantly involved in the production of proinflammatory molecules by instigating the renal podocytes. Preliminary data suggested that the CXCL13/CXCR5 interface can be a promising site for therapeutic agents (Schiffer et al. 2015). Similarly, CXCL12 is also reported to be responsible for the pathogenesis of SLE by facilitating the infiltration of leukocytes. In the chronic case of LN, accumulation of the immune B cells occurs, which is promoted by CXCL12/CXCR4 signaling cascade (Hanaoka et al. 2015). A reduction in the pathobiological aspects of SLE is observed on administration of anti-CXCL12-neutralizing antibody. Thereby, the use of antagonist that limits the activity of CXCL12/CXCR4 interface can be a promising strategy (Balabanian et al. 2003).

CXCL9 is another CXC chemokine that arbitrates SLE pathogenesis upon interacting with its cognate receptor CXCR3. CXCR3 is a major G-protein-coupled receptor (GPCR) that is expressed over the immune cells like T-cells, B-cells, and NK-cells. These immune cells are activated when chemokine CXCL9 binds to its receptor, and facilitates the transmigration of these immune cells in the kidney (Clement et al. 2015). Steinmetz et al. have reported that the mice deficient of CXCR3 receptor has a low infiltration rate of immune cells (macrophages and T cells) in the glomerular region of the kidney, indicating reduction in the pathology indices (Steinmetz et al. 2009). Yu et al. have summarized the involvement of chemokines and their cognate receptors in the pathogenetic aspect of SLE (Yu et al. 2012), as shown in Fig. 4.3. These studies evidenced that targeting the specific protein–protein interactions between the chemokines and their cognate receptors involved in SLE disorders can be a potential strategy for formulating next generation drugs.

4.4.3 Calreticulin (CRT)

Calreticulin is an ER residential multifaceted soluble protein that is established to have numerous functions on both inside as well as outside of ER (Williams 2006). Along with its role in the Ca^{2+} homeostasis, studies indicate that CRT is also

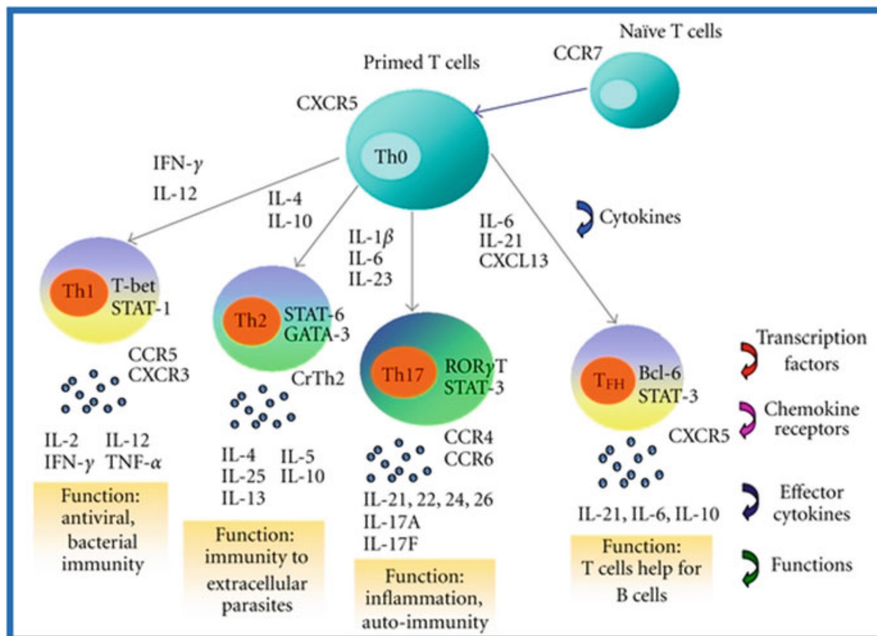

Fig. 4.3 Schematic representation of T cell activation, and role of chemokines and their cognate receptors in different aspects of SLE. (Adapted from Yu et al. 2012)

involved in the pathogenesis of SLE through epitope spreading, inactivation of complement system, and instigation of mediators that facilitate the process of inflammation. Enhanced expression of CRT has been observed in the serum samples collected from the patients of SLE, which is in correlation with the indices of disease progression. Researchers have also proposed that the upregulation of CRT promotes organ damage (Wang et al. 2017). CRT interacts with proteins like E3-Ubiquitin ligase TRIM21 (a nucleoprotein) and cyclophilin B. Further, it also specifically binds to the endoplasmic reticulum-resident protein 57 (ERp57) and glucocorticoid receptor resulting in a series of protein interactome that promotes the initiation and development of SLE. These advocate for the use of CRT inhibitors to target the aforementioned interface that limit/revert the pathogenesis of SLE (Varricchio et al. 2017).

4.5 Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) is an offshoot of recurring sessions of gastrointestinal tract inflammation caused by an abnormal immune response to gut microorganisms. Inflammatory bowel disease is a term used to describe two types of idiopathic intestinal diseases that vary in their location and degree of gut wall

involvement (McDowell and Haseeb 2017). Ulcerative colitis (UC), and Crohn's disease (CD) are the two most predominant inflammatory bowel diseases; both induce digestive issues as well as inflammation in the gastrointestinal system. Symptoms of CD and UC include diarrhea, stomach discomfort, rectal bleeding, and weight loss. Both the disease type is common in adolescents as well as in adults. CD may manifest itself in a variety of phenotypes across the gastrointestinal system, from moderate ileitis to structuring disease to perianal fistulizing sickness. UC, on the other hand, has less phenotypic variation and, by definition, only encompasses the colon (Zhang and Li 2014). To serve the purpose of diagnosis, transmural or superficial patchy granulomatous infiltration and/or acute inflammatory cells are utilized. Both UC and CD have extraintestinal symptoms, but only UC has hematochezia with mucus or pus passing. Fistulas, perianal disease, colonic, and small intestine impairments are common in CD. Both UC and CD feature cryptitis and crypt abscesses, but UC's crypt architecture is more distorted (Baumgart and Sandborn 2007; Yewale et al. 2021). Details of the proteopathic basis of manifestations of IBD are described as follows.

Crohn's Disease (CD)

Crohn's disease is a lingering ailment of the gastrointestinal tract that predominantly affects the terminal ileum, cecum, perianal region, and colon. A weakened immune system, an unbalanced microbiome, genetic predisposition, and environmental elements have all been linked to the initiation and progression of Crohn's disease (Roda et al. 2020). As per the involvement of immune cells are concerned, CD is a disorder characterized by Th1 cells. Patients of CD are reported to have upsurge in levels of proinflammatory cytokines including interferon-gamma (IFN-γ) and IL-17A in their small intestinal inflammation (synthesized by Th1 and Th17 cells, respectively) (Fuss et al. 1996). Furthermore, the Th17 channel (driven by IL-17 generated by Th17 cells) modulates the Th-1 activity (Kolls and Lindén 2004). The IL-17 pathway is activated by innate immune cells and APCs releasing IL-6, IL-23, and transforming growth factor-beta (TGF-β). Th1 cell differentiation is promoted by the expression of transcription factors (STAT4 and T-bet) and cytokine receptors (IL-12R2), which is one of the distinguished features of injured lamina propria in CD patients. Stimulated APCs derived from IL-12 are responsible for activating T-bet, a Th1 master transcription factor (Sartor 2006). Similarly, the expression of IL-23 by ileal dendritic cells promotes the expression intensity of IL-17, resulting in enhanced levels of both IL-17 and IL-23 in the case of CD. As a result, both Th-1 and Th17 pathways are associated in the advancement of CD. Pneumonia and malignancies of the biliary tract, lymphoid, and hematological organs are the major causes of death in CD (Jussila et al. 2014).

Ulcerative Colitis (UC)

Ulcerative colitis is a chronic, idiopathic inflammatory ailment of the colon that most typically causes impairment to the individuals between the ages of 30 and 40. It is marked by recurrent and remitting inflammation in the mucosal region that starts in the rectum and spreads till the proximal regions of the colon (Ungaro et al. 2019).

Patients having UC have upsurge in the chance of acquiring colorectal cancer, which is majorly observed in males as compared to females, predominantly in patients detected with the UC and severe colitis at very young age (Pabla and Schwartz 2020). As per the involvement of immune cells are concerned, UC is mediated by IL-13 which is generated by Th2 cells and nonclassical natural killer T cells (NKT cells) as it works in tandem with TNF-α to control the expression of genes involved in the establishment of tight junction between enteroepithelial cells. Membrane proteins are also disrupted by the IL-13 through accelerated cell death (which is accelerated by TNF-α) and influence the constituent protein of tight junctions (Tindemans et al. 2020).

When commensal microbiota (nonpathogenic bacteria) is detected by TLRs specifically TLR2 and TLR4, it leads to the stimulation of APC (macrophages and dendritic cell). Subsequently, stimulated APCs trigger naive CD4+ T-cells to evolve into diverse subtypes of effector T helper cells including Th2, Th9, and regulatory T cells (Treg) (Ungaro et al. 2016). In the lamina propria of UC patients, the activation of Th2-specific cytokine IL-4 is overshadowed by additional Th2-associated cytokines like IL-5 and IL-13, as well as the Th2 master transcription factor GATA-binding protein 3 (GATA3). As a result, UC is principally a Th2-mediated immunological disease, although given the low levels of IL-4 production, the significance of Th2 cells all together in UC remain unclear. Th9 cells that produce IL-9 are linked to UC because they inhibit mucosal wound healing and disturb the mucus layer's defensive abilities (Nalleweg et al. 2015). Enhanced infiltration of gut-associated lymphocytes to functional gastrointestinal tract and areas of inflammation are caused by high amount of mucosal addressin cell attached molecule-1 (MAdCAM-1), thereby, conforming its plausible involvement in the pathophysiology of IBD. Elevated MAdCAM-1 expression in colon endothelium of diseased rats generated by peptidoglycan-polysaccharide, and consequent mitigation of colitis by anti-MAdCAM-1 antibody, suggest that these molecules are crucially involved in the establishment of colitis (Hokari et al. 2001). Moreover, MAdCAM-1 is observed to be having a significant role in both, UC and CD manifestation. The venules of MAdCAM-1 are substantially more common in the deeper layers of intestinal tissue of CD patients than UC patients, which may explain CD's characteristic transmural inflammation (Arihiro et al. 2002). Apart from these, some other important protein–protein interfaces involved in IBD progression which are discussed below, and some are demonstrated in Chap. 5 as well.

4.5.1 Myeloid-Related Protein 8/14 (MRP8/14) and TLR

Myeloid-related proteins (MRPs), also called as calgranulins are a group of small proteins expressed on the myeloid cells; these proteins are crucial component of innate immune system. Among this group of protein, MRP8 and MRP14 are more prominently responsible for exhibiting the immune response. MRP8 and MRP14 are observed to form a heterodimer that activates them to perform their biological functions (Vogl et al. 2007). In case of inflammatory disorders, Mrp8/14 expression

is linked to the disease activity, most notably inflammatory bowel disease. MRP8/14 is responsible for the deployment of inflammatory cells at the location of injury, along with that an increased expression is also observed after infection (Foell et al. 2007). With the advancement in the modern pathological techniques, MRP8/14 complex has been established as a potent biomarker for the diagnosis of IBD.

MRP8/14 dimer is described to be activating the pathogenesis by modulating the lipopolysaccharide (LPS)-induced secretion of TNF-α. MRP8/14 interacts with other immune TLR proteins, specifically TLR4 and facilitates the lethal endotoxin triggered shocks (Vogl et al. 2007). MRP8 is the component of higher significance in the complex, as it instigates the transmigration of myeloid differentiation factor 88 (Myd88), that in sequence activates the IL-1 receptor-associated kinase-1 (IRAK-1) and NF-κB leading to the upsurge in the expression of immune component TNF-α (Manitz et al. 2003). Considering the fact of high level MRP8/14 intervention in the immune response, targeting/disrupting this complex can be an effective strategy for preventing an unregulated inflammatory response as observed in IBD. Inhibiting the secretion of these inflammatory mediators might be a viable treatment opportunity. On the other hand, inducing a condition of immune tolerance to future inflammatory stimuli by introducing a low dose of MRP8 can be another feasible approach as shown with the TLR4 agonist LPS (Coveney et al. 2015).

4.5.2 NF-E2-Related Factor 2/Kelch-Like ECH-Associated Protein-1

NF-E2-related factor 2 (Nrf2) is a transcription factor that is demonstrated to have the anti-inflammatory and cytoprotective features. Nrf2 binds to the antioxidant responsive element (ARE) region of the particular detoxification and antioxidant genes leading to their upregulation and induction of cellular defense machinery (Aleksunes and Manautou 2007). The activity of Nrf2 is tightly regulated by Kelch-like ECH-associated protein-1 (Keap1). Under basal condition, Keap1 binds to Nrf2 and mediates its ubiquitination, which leads to negative functional regulation of Nrf2. Due to the interaction between Nrf2 and Keap1, the redundant activation of Nrf2 is regulated under normal conditions, whereas, under stress conditions, the disproportionate oxidative agents promote mutations and henceforth the conformational changes in the Keap1 protein. Under such scenario, Nrf2 expresses continuously, thus inducing inflammatory response (Fig. 4.4). Nrf2-mediated protective responses are well reported in the colon region, moreover, it helps to maintain the integrity of intestine by regulating the proinflammatory cytokines and detoxifying enzyme (Lu et al. 2016). Oxidative stress is reported to be the driving force in colitis, the upsurge in the ROS causes damage to the RNA, DNA (nuclear and mitochondrial), lipid, and proteins, which lead to the uncontrolled mutation, unregulated cell growth, and cell death in worst case. Through animal-based experiments, it is validated that the absence of Nrf2 promotes the pathological aspects of colitis (Jena et al. 2012). Defense system of the colonic mucosa is demonstrated to maintain the redox homeostasis and prevents the ROS-induced damage to the colon. Consequently, Nrf2 activation can be a potential strategy to employ the therapeutic effects

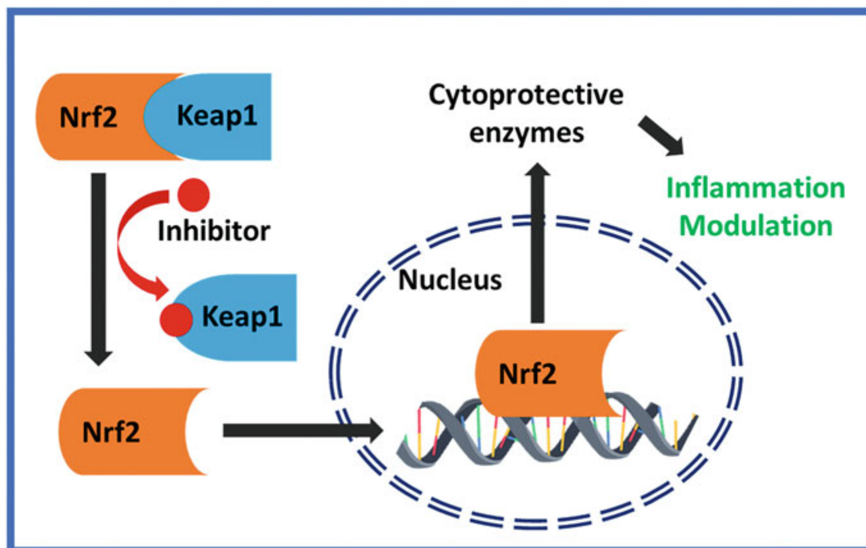

Fig. 4.4 Schematic representing the activity of Keap1 inhibitor. Upon dissociation of Nrf2 from Keap1, it migrates to the nucleus and initiates the expression of cytoprotective enzymes that modulate the course of inflammation

on colitis, considering this, various Nrf2 activators and Nrf2-Keap1 inhibitors have been developed (Abed et al. 2015). For example, compound CPUY192002, CPUY192018, peptide-based inhibitors, and various other molecules as discussed in Chap. 7 in detail have been reported to bind at the interface between Nrf2 and Keap1 inhibiting their association, allowing Nrf2 to remain activated and exert protection against colitis (Lu et al. 2016).

4.5.3 Haptoglobin (Hp)

As the name suggests (Hapto; to bind, globins), haptoglobin is a hemoglobin-binding protein, a part of acute phase proteins family. It interacts with the freely available hemoglobin and inactivates it by generating a stoichiometrically stable composite. It makes the circulation devoid of the free radicals thus detoxifying the plasma, and reducing the chances of kidney damage due to inflammation (di Masi et al. 2020). Human Hp is an acute phase α^2-sialoglycoprotein. Proinflammatory cytokines including IL-1, IL-6, and TNFα boost its synthesis, particularly in hepatocytes. The three basic Hp phenotypes are Hp 1-1, Hp 2-1, and Hp 2-2. Molecular size and structures define Hp phenotypes along with its biological characteristics. The central role of Hp is to bind hemoglobin (Hb). It creates a soluble Hb molecule that bypasses the kidneys, and is broken down in the liver, thereby, saving the kidneys (Buehler and Schaer 2020). Hp also decreases

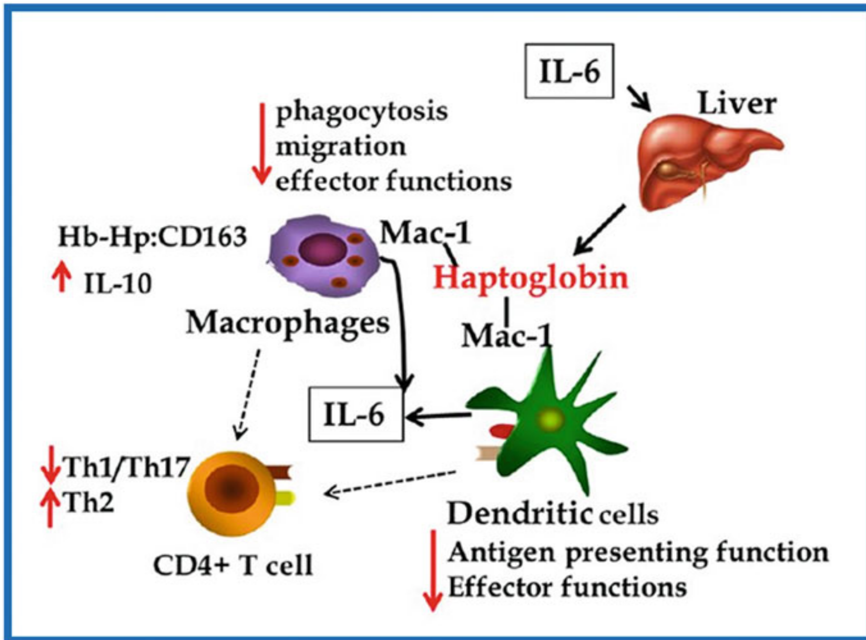

Fig. 4.5 Schematic representing the interaction cascade followed by Hp in regulation of the activities of immune cells. (Adapted from Galicia and Ceuppens 2011)

inflammation by lowering prostaglandins, leukotrienes, and cathepsin B synthesis. It lowers iron-derived reactive oxygen stress, which is coupled to free Hb synthesis. Hp passes through various pathways in its due course of regulating the immune cells as shown in Fig. 4.5.

Due to its polymeric composition, Hp 2-2 has the least antioxidant and anti-inflammatory activity. As HP is a protein that modulates the immune reaction and decreases oxidative stress, its participation in the inflammatory processes engaged in IBD and other immunological disorders such as arthritis may be critical (Levy et al. 2010). Marquez et al. reported that, Hp protein plays a protective role in the intestine. It has been examined that Hp knockout mice developed severe symptoms of IBD, specifically due to the upregulation in the concentration of IL-17. It is also worth mentioning that the concentration of Hp2 is higher in the serum of IBD infected model in comparison to that of a healthy group (Marquez et al. 2012).

4.5.4 Ring Finger Protein 5/S100A8

Ring finger protein 5 (RNF5) is a ubiquitin ligase that is associated with the endoplasmic reticulum. This protein is one of the key players in the removal of misfolded proteins by Endoplasmic Reticulum-Associated Degradation (ERAD) for

a sustained protein homeostasis in the cell. As a matter of function, RNF5 identifies the misfolded proteins and instigates the process of ubiquitination, which facilitate the proteasome-dependent degradation of these misfolded proteins (Tcherpakov et al. 2009). S100A8, an interactor of RNF5 belongs to the S100 protein family that is majorly expressed in the neutrophils and monocytes. S100A8 are the damage-associated molecular patterns (DAMPs) which are released at the time of tissue damage, and they interact with the pattern recognition receptors (PRRs) located on the immune cells to prompt the inflammatory response. It is worth mentioning that S100A8 is crucially involved in the pathogenesis of IBD (Boyapati et al. 2016). RNF5 is reported to limit inflammation in the intestine by regulating the stability of S100A8 protein. Moreover, RNF5/S100A8 complex is also observed to control microbial (viral and/or bacterial) contamination by governing the immune sensing machinery (Fujita et al. 2018). Owing to the crucial pathological role of RFN5/S100A8 interface in development and progression of IBD, it can be a promising therapeutic target in the treatment of ulcerative colitis.

4.6 Type-1 Diabetes (T1D)

Type-1 diabetes (T1D) is a multifaceted autoimmune disorder initiated by genetic and environmental factors leading to the irregularity in the expression and functioning (insulin production) of pancreatic β cells. T1D can be diagnosed by analyzing the serum autoantibody produced against the pancreatic β cell antigens (Atkinson 2012). International Diabetes Federation (IDF) has predicted that, on a global scale by the year 2030, approximately 550 million people will have T1D (Saeedi et al. 2019). It is well evidenced that both innate and adaptive immune responses are involved in the pathogenesis of T1D; T-cell-mediated degradation of pancreatic β cell is one of the distinguished features of T1D. Cells that are majorly involved in the destruction of β cells include T cells (CD4+ and CD8+), B cells, DC, NK cells, and antigen-presenting cells (Tai et al. 2016). Interestingly, along with the endocrine pancreatic cells, exocrine pancreatic cells are also affected in the due course of disease initiation and development (Campbell-Thompson et al. 2016). In the initial days of T1D research it was believed that, due to the abnormal functioning of the immune system, β cells completely lose their ability to produce insulin, although the functioning of pancreatic β cells remains intact up to 40% (Leete et al. 2016). The involvement of more than 50 loci supports the initiative of using the proteins and their associated interaction- interfaces as the target for the treatment of this disease (Pociot and Lernmark 2016). Currently, the strategy of suppressing the unwanted immune response is being explored. By targeting these proteins and their interactions, not only one can downregulate the immune response, but also can promote proper functioning of β cells (Atkinson et al. 2019). Some of these major proteins involved are explained below.

4.6.1 Protein Tyrosine Phosphatase (PTPs)

Protein tyrosine phosphorylation is a crucial regulatory mechanism that is involved in multiple aspects of cellular functioning (Tonks 2013). This process is catalyzed by the enzyme protein tyrosine phosphatases that are involved in the reversal of protein tyrosine kinase activity by dephosphorylating the tyrosine residue of the proteins. Dysregulation in the functioning of this enzyme is described in various diseases apart from autoimmune disorders that include cancer and neurological diseases (He et al. 2014). Upon association with the IFN receptors expressed on pancreatic β cell, tyrosine kinase JAK1 and JAK2 is activated which sequentially leads to the phosphorylation of STAT1. The STAT1 in its phosphorylated state forms a dimer and is transmigrated to the nucleus, where it stimulates the production of chemokines along with the proapoptotic molecules (Thomas et al. 2009). Owing to the fact of PTPs involved in the activation of various protein–protein interfaces, researchers have shown that inhibiting these proteins stands a potential approach for the treatment of T1D. A very low dose of streptozotocin that targets the STAT1 and leads to its disruption, defends the β cells from the unwanted immune cells activated against it (Gysemans et al. 2005).

4.6.2 Peroxisome Proliferator-Activated Receptors (PPARs)

Glucose and lipid metabolism are undoubtedly crucial cellular activities, arbitrated by a transcription factor peroxisome proliferator-activated receptor (PPARs). PPARs are ubiquitously expressed on all the cells including pancreatic β cells and other immune cells that are responsible for the secretion of insulin and in regulating the differentiation of T cells (Holm et al. 2020). Upon interaction with its ligands (polyunsaturated long-chain fatty acids and arachidonic acid derivatives), PPARs migrate to the nucleus and bind to the retinoid X receptor (RXR). Subsequently, PPAR-RXR complex forms a protein–protein interactome with peroxisome proliferator hormone response elements (PPREs), arbitrates the expression of target genes that are crucial for the glucose/lipid metabolism and activation/survival of T cells, and significantly facilitates the T1D disease management (Christofides et al. 2021; Hong et al. 2018). PPARs are expressed in three isoforms namely, PPARα, PPARβ/δ, and PPARγ; out of which, polymorphism in PPARβ/δ and PPARγ is described to be crucial for pathogenesis of T1D and induction of chronic response (Holm et al. 2018). Owing to the facts discussed, inhibition of PPARs-RAR-PPREs protein–protein interactome can be an effective therapeutic against T1D. Use of antagonists that bind PPARs instead of the corresponding ligands to hinder the activity of the transcription is much explored nowadays. For example, Troglitazone, an agonist of PPARγ has been described in improving the function of pancreatic β cells by preventing the alterations in the mitochondrial functioning (Jia and Otsuki 2002). Similarly, the agonist of PPARβ/δ that includes GW501516 has also been established to improve the functioning of β cells and the tolerance for glucose and glucose-stimulated insulin-secretion (GSIS) (Tang et al. 2013).

4.6.3 Sodium Glucose Transporter (SGLTs)

SGLTs are the sodium-dependent glucose transporter membrane proteins that are majorly expressed in the kidney and are entailed in the transmigration of glucose, amino acids, and other essentials through the membrane of epithelium of intestine and renal tubules (Dellepiane et al. 2018). SGLTs possess very high capacity of membrane transportation of glucose, but lack affinity resulting in the transportation of vitamins and inositol as well (Scheepers et al. 2004). Glucose is reabsorbed in the kidney through the SGLTs, specifically, SGLT2 (an isoform out of six) is responsible for up to 90% of the reabsorbed glucose making it a potential point of treatment of T1D (Zinman et al. 2015). The activity of the SGLT2 protein is observed to be positively regulated through its interaction to the protein called membrane-associated protein (MAP17). SGLT2-MAP17 complex leads to the activation of SGLT2 protein, and is also demonstrated to be associated with the pathogenesis of T1D (Coady et al. 2017). Numerous drug molecules have been explored which can inhibit the activity of SGLTs, and are known as SGLTi. These molecules can be used as supplementary to insulin and facilitate the improvement in the glycemic control. Phlorizin was the first SGLTi, discovered in nineteenth century, but has not been approved to be used on humans yet (Ehrenkranz et al. 2005). Recently, Sotagliflozin has been reported to have inhibitory activity against SGLT2, that directs improved glycemic control in the treated mice with condensed menace of hypoglycemia (Powell et al. 2015). In addition to this, Empagliflozin and Canagliflozin, inhibitors of SGLT are also in the clinical trial phase for the treatment of T1D (Dellepiane et al. 2018).

4.7 Psoriasis

Psoriasis is a chronic multifaceted autoimmune ailment affecting 2–5% of the global human population. This is an inflamed skin condition specifically distinguished by the scaly patches of skin (Dogra and Mahajan 2016). The condition of psoriasis is induced by the hyperproliferative epidermal cells which result from immature generation of keratinocytes along with the incomplete salvation of the nucleus in the outermost layer of the skin (Wikramanayake et al. 2014). An alteration in the basal cell division is observed in comparison to the normal cells. It has been reported that psoriasis induces and/or coexists with diseases like diabetes, hypertension, coronary heart diseases, neurological disorders, gastrointestinal disorders, chronic obstructive pulmonary disorders (COPD), etc. (Srivastava et al. 2021). Psoriasis pathology is characterized by the extensive growth of keratinocytes, which promotes the thickening of epidermal layer and aggregation of inflammatory exudates like neutrophils, macrophages, T cells (CD4+ and CD8+) in the dermis region that is crucial for the initiation and progression of psoriasis. Dendritic cells (DC) are reported to be significantly involved in the initiation of psoriasis (Lowes et al. 2007). Further, the driving element for the psoriatic inflammation majorly consists of Th17 cells and Th1 cells. Functioning of these cells are arbitrated by cytokines

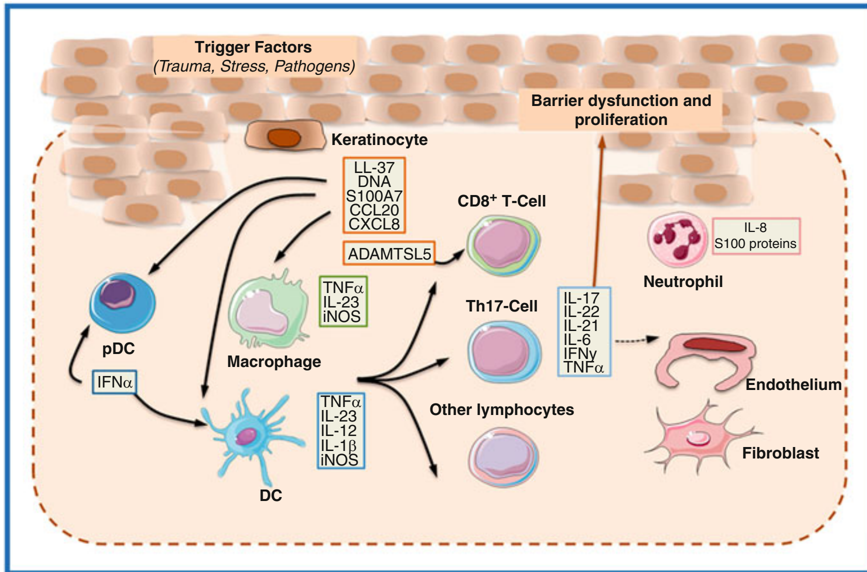

Fig. 4.6 Immune cells involved in the pathogenesis of psoriasis. (Adapted from Rendon and Schäkel 2019)

like IL-23, IL-17, and TNF-α (Rendon and Schäkel 2019). IL17 cytokine has six members, IL-17A-F, out of which only IL-17A and IL-17F have significance in the pathogenesis of psoriasis. These variants of IL-17 are described to activate the kinase-mediated intracellular signaling cascades that include MAPK, extracellular signaling-regulated kinases (ERK), I-kappa B kinase (IKK), and JAK-STAT pathways (Lee et al. 2015). Furthermore, various immune cells are also responsible for the psoriatic pathogenesis as diagrammatically represented in Fig. 4.6. The machinery of cell cycle is governed by numerous proteins expressed in the living cells. On this note, there are structural and functional proteins, and their interacting protein partners accountable for the differentiation and proliferation of epidermal keratinocytes, which regulate the host immune response. These proteins include, TNF-like weak inducer of apoptosis (TWEAK), Aquaporin-3 (AQP3), and CARD14/CARMA2 (Yadav et al. 2018). Drugs targeting these proteins, or their interaction interfaces can be a prospective treatment strategy for the different aspects of psoriasis.

4.7.1 TNF-Like Weak Inducer of Apoptosis/Fibroblast Growth Factor-Inducible 14

TNF-like weak inducer of apoptosis (TWEAK) is a protein that comes under the TNF superfamily. It is crucial for the appropriate cellular functioning through its specific association with the fibroblast growth factor-inducible 14 (Fn14) receptor.

Fn14/TWEAK signaling cascade is reported to be highly expressed in the psoriatic patients that facilitate the differentiation of keratinocytes (Sabour Alaoui et al. 2012). Bilgiç et al. established in his research about the increased expression of TWEAK along with the other immune proteins including IL-23, IL-06, TNF-α, it is also observed that these chemokines are in synergy with the TWEAK and helps in the advancement of psoriatic pathogenesis. Furthermore, TWEAK is also involved in IL17 singling cascade (Bilgiç et al. 2016). Owing to these findings, inhibition of TWEAK can be an effective strategy in the treatment of psoriasis, as FN14 receptor is not expressed on the T and B cells. Such strategy is very promising, as it will not interfere with the standard immune responses (Xia et al. 2011).

4.7.2 Aquaporin-3 (AQP3)

Aquaporins (AQPs) are the intrinsic proteins which are responsible for the water channeling through the membrane. There are 13 different AQPs reported in mammals till date, most of which are reported to be located in the kidney (Nielsen et al. 2002). Aquaglycoprotein 3 (AQP3) is the most studied aquaporin in psoriasis. AQP3 is majorly related to the functioning of the epidermal cells of the skin that facilitate the transportation of water and glycerol, the activity of AQP3 is reported to be regulated by the histone deacetylase (HDAC). HDAC regulates the activity of AQP3 through acetylation, and also by controlling the transcriptional activity of p53 and associated members (Hara et al. 2002). In various studies, it has been established that any aberration in this protein promotes the skin impairments and delayed wound healing. Moreover, AQP3/HDAC protein axis is also observed to be desirable in the differentiation and proliferation of keratinocytes (Hara-Chikuma and Verkman 2008). AQP3 has been explored, as it is widely expressed in the skin cells, and reported that its concentration decreases in the psoriasis patients (Lee et al. 2012). These evidences indicate that, targeting the malfunctioning of AQP3, and enhancing its expression can be an effective approach for reducing the symptoms of psoriasis (Yadav et al. 2018).

4.7.3 Caspase Recruitment Domain/Caspase-Recruited Membrane-Associated Protein

Caspase-recruited membrane-associated protein (CARMA proteins) belongs to the superfamily of scaffold molecules that are significantly involved in the regulation of immune cell recruitment at the site of inflammation. CARMA proteins are also involved in tissue homeostasis and alteration in the GPCR signaling cascade (Blonska and Lin 2011). These proteins are caspase recruitment domain (CARD) containing proteins that exist in three isoforms: CARD11/CARMA1, CARD14/CARMA2, and CARD10/CARMA3 (Zotti et al. 2018). On the premise, CARD14/CARMA2 performs its signaling function through CBM complex comprising of CARD14/CARMA2, BCL10, and MALT1, where BCL10 acts as an adapter protein

and MALT1 as a protease. Therefore, it is legitimate to speculate that CBM complex produces the molecules facilitating the activation of NF-κβ (Afonina et al. 2016). Mutation in CARD14/CARMA2 upregulates the inflammatory transcript in keratinocytes that include CXCL8, CCL20, and IL6. CARD14/CARMA2 also induces signaling through IL-23/IL-17 axis that promotes the establishment of psoriasis symptoms. Inhibitors of CARD14/CARMA2 and associated proteins (BCL10 and MALT1) can be accounted as effective drug molecules are able to reduce inflammatory responses and alleviate psoriatic ailments (Lee et al. 2018).

4.8 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a lingering autoimmune disorder, reported in approximately 1% of the total world population and is principally observed in females more than in males. This condition typically occurs between the 35–60 years of age, with phases of abstinence and relapse. Juvenile RA (JRA), similar to RA, affects the young children of 16 year or younger but the rheumatoid factor is not present in the JRA (Guo et al. 2018). Some of the prevalent characteristics of RA include, prolonged morning stiffness, exhaustion, fever, loss of weight, sensitive, inflamed, and warmed joints, nodules of rheumatoid under the skin (Bullock et al. 2018). It was also well distinguished by bone and cartilage destruction above joints, as well as knocking off the joint and rigidity (Nithyashree and Deveswaran 2020). In the advanced case of the disease, synovial membrane is invaded by the immune cells that leads to the formation of pannus (tissue granules), thereby, leading to systemic inflammation of the joints, persistent synovitis, tissue impairment, and inflammation because of an imbalance between the autoimmune cells (Girdler et al. 2020). There appears to be a significant interaction amongst the components of both adaptive as well as innate immune system in the case of RA. Apart from rheumatoid arthritis synovial fluid (RASF), immune cells like B and T lymphocytes, mast cells, neutrophils are also triggered in the case of RA (Scherer et al. 2020). Numerous complicated interplays take place between the proteins that are not well studied and this lack of information about the pathogenesis of RA is the main obstruction in the path of therapeutic developments (Kim et al. 2006). In the sections below, different proteins involved in the RA pathogenesis and their interacting partners along with their pathological aspects have been presented.

4.8.1 Galectins (GL)

Galectins are the carbohydrate (β-galactoside)-binding proteins that are evolutionary preserved in animals. There are 15 members of galectin reported till date that are categorized into three sub groups namely: prototype galectin (GL-1, -2, -5, -7, -10, -11, -13, -14, and -15), tandem repeats type galectin (GL-4, -6, -8, -9, -12), and chimera galectins (GL-3) that are ubiquitously expressed in almost all the immune cells (Salamanna et al. 2019). Galectin, through its cognate receptor arbitrates

intra- as well as extracellular signaling cascades, and also regulates the interaction between a cell and the extracellular matrix (ECM) (He and Baum 2006). There are piles of evidences which support the fact that galectins (GL-1, -2, -3, -8, and -9) regulate the pathophysiology of RA in both negative and positive manner; GL-1, -9, and -2 have anti-inflammatory effects and are negative regulator of inflammation, whereas, GL-3 and GL-8 are proinflammatory galectins (Li et al. 2013). Galectins are secreted in the inflamed synovial region, owing to the upsurge in the concentration of proinflammatory galectin, it mediates the initiation and progression of RA (Toscano et al. 2018). In accordance with their role, either the derivatives of anti-inflammatory galectins or inhibitors of proinflammatory galectins can be potential therapeutic approaches for the treatment of RA.

GL-9 is the major galectin involved in the pathogenesis of RA. GL-9 interacts with the glycosylated co-stimulatory transmembrane receptor called 4-1BB and induces signaling cascade for the clonal expansion and generation of CD4+ and CD8+ T cells along with the cytokines in RA (Nielsen et al. 2016). Many attempts have been made to inhibit the signaling arbitrated through this axis. Recently Nielsen et al. have reported the use of GL-3 as the competitive binder of 4-1BB that exerts no RA pathogenesis upon complex formation and reverts the reactions observed by Gal-9 (Nielsen et al. 2022). In contrast, the involvement of GL-3 and its cognate GL-3-binding protein (GL3BP) in the pathogenesis of RA has been also reported (Mendez-Huergo et al. 2019). Considering their complex roles, galectins, and their cognate-binding proteins in RA, they can be considered as potential therapeutic targets for exerting the health-promoting effects.

4.8.2 Connective Tissue Growth Factors (CTGF)

Connective tissue growth factors are the proteins of cellular communication network (CCN) family also called as CCN2. This protein is synthesized by the endothelial cells that are crucially involved in numerous processes of cellular functioning that include angiogenesis, inflammation, wound healing, etc. (Ramazani et al. 2018). Till date, no specific receptor for the CTGF has been reported, but it directly interacts with the integrins and other proteins of extracellular matrix to exert its biological functions. Transforming growth factor beta (TGF-β) is the very first identified binding protein of CTGF which induces its expression. Further, CTGF is also reported to interact with the cell surface integrins and initiate the process of cell adhesion, migration, and other intracellular signaling cascades (Arnott et al. 2011). It is evident that CTGF is responsible for the induction of inflammation in the person having RA. For the very first time, Nozawa et al. (2009) reported the presence of CTGF in the sera of RA patients, later, this fact was also supported by the work of Ding et al. (2016). Further, the findings of Yang et al. (2017) have demonstrated that the serum of RA patient is approximately 90% sensitive, hence it can be a potential marker for the diagnosis of RA. In RA patients, the production of CTGF in synovium is reported to be triggered by TGF-β. CTGF having the characteristics of cytokines, interacts with membrane proteins and acts as the regulator of TGF-β

(Auréal et al. 2020). In addition, the process of osteoclastogenesis is also triggered by the CTGF; in a mice-based experiment it has been shown that, by neutralizing the antibody generated against CTGF, the effect of abnormal osteoclastogenesis can be regulated (Nozawa et al. 2013). However, the protein interactome background of this conclusion is still to be explored.

4.8.3 Calreticulin (CRT)

Calreticulin is calcium-binding protein widely expressed in all cell types. It was initially reported to be resident of endoplasmic reticulum and has the features of molecular chaperons. A handful of evidences from recent studies have established the fact that CRT synthesized extracellularly is involved in the signaling cascade, arbitrating the process of apoptosis, immune response, and adhesion of cells (Gold et al. 2010). As discussed by Tarr et al., the upsurge in the concentration of CRT has been observed in the RA patients. CRT interacts to the Fas ligand (FasL), an extracellular inducer of apoptosis, and inhibits the apoptotic activity of FasL and mediates the degradation of the Jurkat T cells, thereby, facilitating the infiltration of proinflammatory immune cells in the joints of RA patients (Tarr et al. 2010). The autoantibodies generated against CRT, hamper the interaction between CRT and FasL and neutralize the apoptosis of immune cells in the RA. Hence, interface between the CRT and transmembrane FasL protein can be a potential therapeutic target to neutralize the inflammatory responses.

4.9 Conclusions

Autoimmunity, a situation where the body stops recognizing its own cells/tissues and initiates inflammatory signals to eradicate the same, paves the basis of autoimmune diseases. A handful of PPI interaction networks are available in the literature carrying out the autoimmune reactions culminating in the plethora of cytokines instigating the inflammatory responses. The current chapter described most of the proteins involved in the protein–protein network associated with the majority of what is currently known as autoimmune diseases. Multiple overexpressed proteins in proteomics study have been identified and can be further explored to gain knowledge about sensitive biomarkers for better prognosis of varied autoimmune diseases. Utilizing the information of molecular mechanism of these protein–protein interactions, attempts can be made to target the PPI networks involving proteins such as T-cell receptor, CTLA4, ICAM1, MHCs, myelin basic protein, peptidylarginine deiminases, Nrf2, TWEAK, etc. Our understanding of the involvement of autoimmune diseases has been getting enriched over the years, consolidating the expectations of constructing a better treatment and diagnosis regimen in the near future by targeting the associated PPIs.

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Protein-Protein Interactions in Host–Pathogen Interactions

5

5.1 Introduction to PPIs in Host–Pathogen Interactions

Human body is a home to various microbial populations having both positive and negative impacts on the host. In the past few centuries, several diseases have emerged as deadly and disease-causing pathogens such as bacteria, viruses, fungus, and parasites have been known to participate in this havoc now and then. Thus, investigating pathogen-associated protein–protein interactions (PPI) and molecular networks are of great interest in defining the causality and cure for the infectious diseases. Modern techniques such as whole-genome sequencing, proteomics, and metabolomics have been of immense help in understanding molecular changes at the gene level. The effect, survival, and host defense mechanisms against microbes all together rely on the interaction of host and microbe with each other. These interactions determine the sustenance of microorganisms in a host body and are defined as Host–Pathogen interactions (HPIs). The HPIs are spectacular and have been studied at various levels ranging from molecular to population level in order to describe the mechanisms behind pathogenicity. The spread and severity of infections majorly depend on the extent of HPIs. From invasion, proliferation to disarming host defense mechanisms, every process involves protein–protein interaction between host and pathogen (Casadevall and Pirofski 2001). Host–Pathogen interactions can be divided into four stages: (a) pathogen invasion; (b) evasion; (c) proliferation; and (d) host immune response. As each microorganism has its distinct characteristics and a piece of different machinery at various levels, the action and reaction of the associated pathogen and the host are unique, and sometimes discontinuous (Southwood and Ranganathan 2019).

Moreover, from the evolutionary perspective, studying HPIs has aided in analyzing the fundamental nature of microorganisms, and how they evolve into novel strains. In recent years, the world has come across deadly epidemics resulting from zoonotic jumps, multiresistant strains of pathogens, especially viruses and bacteria, and to some extent the lifestyle choices (Sen et al. 2016). These novel

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pathogenic strains are often a product of the ever-evolving evolutionary arms race between the host defense and the pathogenesis. Elucidating PPIs involved in Host–Pathogen interactions provides an insightful understanding of disease progression and reciprocation by the host (Ryan and Matthews 2005). Several databases and bioinformatics tools have been developed to provide a cohesive library of all the pathways and biological networks involved in infections (Zhou et al. 2020). Overall, investigating the protein interactome associated with disease progression has promoted the development of effective diagnostic and treatment methods; and in understanding the knits and grits of how natural selection is responsible in elevating the specificity and sensitivity of hosts and pathogens toward each other (Zhang and Cheng 2022). Due to the unique nature of pathogenesis, host–pathogen PPIs are critical targets in development of drugs, vaccines, and diagnostic kits (Vasala et al. 2020). Investigating host–pathogen interactions also gives a precise purview of the molecular mechanisms involved in immune responses and enhances the therapeutic efficacy. This chapter aims to provide a brief account of proteins and HPIs involved in various microbial infections, and their relevance in the disease austerity.

5.2 Microbial Pathophysiology

Both pathogen and host physiology are culpable for the pathogenesis and development of disease. As mentioned earlier, the human body is a reservoir of microflora, and a disease originates only if a microorganism is able to disrupt the homeostasis of human physiology. The pathways and interaction involved in various stages as mentioned above overlap, as the host immune response and disease progression are simultaneous processes (Wolfe et al. 2007). Further due to similarity in the structure of several pathogens and presence of a variety of strains, the protein–protein interactions involved in host–pathogen interactions are broadly studied and targeted according to these stages. A brief information of the mechanisms involved in the four stages with examples is discussed as follows.

5.2.1 Adhesion and Invasion of Host System

The entry of a microbe into the human body is hindered by anatomical barriers such as skin, hair, and mucosal glands. However, several opportunistic bacteria and other pathogens invade the human body when there is a breach in any barrier, then start multiplying and growing exponentially. For a successful infection, the microbe must follow three rules: (a) first is adherence to tissue, (b) second is the secretion of toxins, and (c) the last is blending virulence factors into the host cell milieu (Lee and Schneewind 2001). Each pathogen invades hosts in a distinct manner and at a specific location in the human body. For example, the bacterial pathogen, *Vibrio cholerae*, adheres to intestinal epithelial cells with the help of surface adhesin called TCP pili and secretes toxins (Lee and Schneewind 2001). Fungal and bacterial pathogens adhere to host tissue and use effector proteins, secretion systems, and

toxins to create an optimal growth environment. While viral pathogens use the process of lysogeny, lysis, and endosome formation with the help of membrane fusion proteins to invade host cells. In comparison to bacteria and fungi, the viral invasion machinery is much simpler. The fusion proteins interact with the host cell membrane and commence the transfection of viral genetic material into the host cell, while fungal and bacterial entry involves effector protein complexes. However, rather than being injected into the host cells, the fungal effector proteins are located on the hyphal surface of pathogen and are presented to host cell receptors that induce modifications in the cell milieu (Southwood and Ranganathan 2019).

The mechanics involved in pathogenic invasions primarily depend on the interaction of membrane proteins of the host and pathogen. Hence, several post-translational modifications play a significant role in host–pathogen interactions. One of the extensively studied post-translational modifications involving HPIs is glycosylation (Lin et al. 2020). As host membrane proteins are heavily glycosylated, several bacterial pathogens often target membrane glycoproteins. The extremity of pathogenicity is also affected by degree of glycosylation (Lin et al. 2020). The invasion process in various bacteria and viruses takes advantage of adhesin-host glycan interactions for adhesion of pathogens. For instance, interaction between FimH adhesin (uropathogenic *E. coli*) and uroplakins, an α-D-mannosylated glycoprotein leads to initiation of urinary tract infections (UTIs) (Foroogh et al. 2021). Similarly, tetraspannins such as CD151 and CD81 are involved papillomavirus, *E. coli*, *S. typhi*, and *L. monocytogenes* infections respectively (Scheffer et al. 2014; Karam et al. 2020).

5.2.2 Evasion of Host System

Upon entering the host system, the pivotal focus of the pathogen is to dodge the host immune system to proliferate. The mechanisms employed for bypassing the immune system are more or less same for all infectious agents. This includes calibrating the surroundings and quorum sensing, impeding macrophage activity, subversion of defense activity (Lachmann 2002), impair molecular crosstalk, and autoimmune responses. Some of the most studied evasion mechanisms for pathogens including, bacterial, parasitic, and viral infections are attacking the NF-κB pathway (Bouayad 2020), inhibition of hub proteins, and IL (interleukin) production as discussed below (Leseigneur et al. 2020; Sen et al. 2016).

The first and foremost action taken by pathogens, after intrusion, is to alter the turf for ambient growth. This alteration is bought about by modifying the extracellular as well as intracellular physiological conditions by secreting endo/exotoxins. *Staphylococcus aureus*, a gram-positive bacterium, releases two kinds of exotoxins namely, pyrogenic toxin superantigens (PTSAg) and hemolysins, which are involved in toxic shock syndrome and food poisoning (Hu et al. 2017; Dietrich et al. 2021). Similarly, *Clostridium botulinum* produces a class of neurotoxins known as botulinum toxins under anaerobic conditions that causes food poisoning and inhibits the secretion of acetylcholine by cleaving synaptic vesicle fusion proteins like syntaxin and

synaptobrevin, leading to paralysis (Hanson and Stevens 2000; Zhang et al. 2017). Botulinum toxins are also used for treating muscular diseases and for cosmetic applications (Zhang et al. 2017). Bacteria such as *Listeria monocytogenes*, *Clostridium*, and *Staphylococcus aureus*, utilize toxins to subvert phagocytic activity, molecular crosstalk, and use macrophage as a vehicle to move from one part of the host to another (Leseigneur et al. 2020).

Another strategy of bacterial subversion/evasion from phagocytic immune cell is capsule formation. The capsules are made up of polysaccharides and lipids, which encapsulate and protect the bacterial population from opsonization by making them invisible to the host immune system. Quorum sensing and biofilm formation play a vital role in this process. For instance, in *Salmonella* species, the PhoP/PhoQ sensor system alters lipid A and downregulates the TLR4 activation and NF-κB expression (Finlay and McFadden 2006). Similarly, AHL-based quorum sensing (QS) systems of *Pseudomonas aeruginosa* evade immune response by biofilm formation and excreting out the antimicrobial components (Alhede et al. 2014). Another trick used by bacteria to steer clear of immune response is antigenic variation. Antigenic variation is one of the vastly studied mechanisms of deceiving the host immune system and is a major factor in the emergence of novel strains of pathogens. These variations are an outcome of point mutations and evolutionary mechanisms adopted by microorganisms for their sustenance (Deitsch et al. 2009). The molecular mechanisms behind antigenic variations involve varied sub-populations of an antigen, polygenic expression, and variable regions/epitopes present on antigen surfaces. The best-studied cases of antigenic variation and its impact on host–pathogen interaction have been observed in Gonorrhea, *Neisseria*-mediated meningitis, and tuberculosis (Palmer et al. 2016; Ernst 2017).

The viral machinery exploits similar pathways and phenomenon for evasion as bacteria. However, the mechanistic properties of the process are marginally different compared to bacterial evasion mechanisms. For instance, while bacteria modulate its niche with the aid of toxins and proteases, the viral pathogens are aided with cell surface proteins that mimic the host receptor or ligand functions. In a similar fashion, the subversion of phagocytic activity by virus targets and kills lymphocyte cells such as macrophages and NK cells or stimulates autoimmune responses rather than escaping the phagosomal fusion. Nonetheless, both bacteria and viruses subvert phagocytes via interaction with inflammatory proteins such as interleukins (ILs) for blocking the inflammatory pathways (Sarantis and Grinstein 2012). Other pathogens such as fungi, protozoa, and helminths also follow the similar evasion mechanisms. For example, the hyphal form of *Candida albicans*, a fungal pathogen, prevents phagocytosis as it is not internalized efficiently. Further *Candida neoformans* are resistant to phagocytosis due to the large polyploid cell size called Titan or giant cells (Collette and Lorenz 2011). Another immunomodulatory mechanism which is employed by most of the pathogens is the subversion of the complement system. The complement system recognizes microbial membrane proteins and enhances the antigen presentation for effective phagocytosis and degradation. However, pathogens have evolved several ways to subvert complement systems. For example, helminths and retroviruses acquire host coating, while viral

and protozoal pathogens exhibit antigenic variations to destabilize the complement system (Lachmann 2002). The mechanisms of abovementioned and several other escape methods adapted by bacterial, viral, and other pathogens are elaborated in later sections.

5.2.3 Microbial Proliferation in Host

The extent and process of proliferation vary between pathogens and define the intensity of infection. Considering the nanoscopic size and concise genome of pathogens, they exploit the host replication machinery and other protein factors in several ways. Pathogens such as viruses commandeer host cells for growth and share a highly dependent relationship with the host than other pathogens. The entire host replication machinery and cellular components are at the virus' disposal; and unknowingly, the host also acts as a vector for gene transfer between viruses. Brass et al., identified approximately 213 host proteins as significant factors for replication of HIV-1 virus (Southwood and Ranganathan 2019; Achuthan et al. 2018). While bacteria and fungi thrive in humans either by adhering or entering the host cells (Ribet and Cossart 2015). For example, *Salmonella enterica* serovar *Typhimurium* survives inside host epithelial cells and macrophages (Raghunathan et al. 2009). However, fungal pathogens such as *Candida* proliferates by attaching to the mucosal and epithelial membrane tissues in their unicellular or hyphal form (Pappas et al. 2018). Various proliferative mechanisms involved in proliferation and colonization of microbes are discussed below.

5.2.3.1 Intracellular Colonization

Subverting host immune response by intracellular colonization is a key survival technique deployed by pathogens. For an effective intracellular entry and colonization, bacteria such as *Salmonella enterica*, *Shigella*, and *Listeria* spp. interact with actin cytoskeleton, microtubules, and intracellular pathways (Rottner et al. 2005; Da Silva et al. 2012). Interaction with the cellular cytoskeletal system benefits bacterial pathogens by giving them motility and tensile support for their optimum growth. This often takes place by further regulation of G protein signaling by the effector proteins. In *S. enterica*, the effector proteins SopE and SopE2 are injected into the host cellular system by the T3SS system. These effector proteins function similarly to guanine nucleotide exchange factors (GEFs) and activate CDC42 and the RAC G proteins (Friebel et al. 2001). Upon activation, the host cell is signaled to engulf the bacteria by the formation of actin-rich membrane ruffles. As soon as the bacterial cell is engulfed, they manipulate the cytoskeletal system by nucleation of actin. In bacterial species like *Listeria* spp., bacterial protein ActA relays the signal for actin nucleation by interacting with the Arp2/3 complex and vasodilator-stimulated phosphoprotein (VASP) (Laurent et al. 1999; Zalevsky et al. 2001). Extracellular pathogens such as enterohemorrhagic and enteropathogenic *E. coli* (EHEC and EPEC) also utilize actin during their course of infection. The enteropathogenic *E. coli* secretes the Tir and noncatalytic region of tyrosine kinase (Nck) protein

into the host cell by the T3SS system, and modifies the actin filament at the site of bacterial adhesion using the Arp2/3 and N-WASP signaling pathway. While enterohemorrhagic *E. coli* recruits Tir and EspFu protein to mediate the polymerization of actin and form a foundation for bacterial support on the host cell membrane, the base formed due to the interaction of bacterial protein and actin filaments is known as pedestals. The pedestals are mobile in nature and are complexed with actin-associated proteins such as contractin, growth factor-bound protein (GRB2), SRC homology 2 domain-containing transforming protein C (SHC), vinculin, and zyxin (Bhavsar et al. 2007). Interaction of pedestals with the proteins mentioned above mediate and regulate intracellular signaling at the time of infection.

Other cytoskeletal filaments such as microtubules are also commonly targeted by the abovementioned pathogens as microtubules are an essential part of cellular cargo units. Unlike actin filaments, the virulence proteins of pathogenic bacteria tend to delocalize the microtubule assembly at the site of microbial invasion. For example, the virA and EspG proteins of *Shigella* spp. and EPEC bind to α- and β-tubulin respectively, to stimulate microtubules' degradation (Hardwidge et al. 2005). However, some pathogens like *Campylobacter jejuni* utilize polar properties of kinesin and dynein for cellular internalization. Kinesins and dynein are microtubule-associated motor proteins that aid cellular transport and are exploited by *C. jejuni* for cellular entry (Bhavsar et al. 2007). As soon as a pathogen can freely interact with the cytosolic resources of the host cell, it interferes and mimics the intracellular signaling pathways. The main target for disrupting cellular signaling is by interfering with the protein kinases and phosphatase-mediated phosphorylation cascades. For example, the YpkA produced by *Yersinia* spp. imitates the function of serine/threonine kinases and modulates actin filaments by direct interaction with GTPase RAC1 (Fischer and Glass 2019). In a similar fashion, the regulation of gene expression by pathogens also plays an essential role in the growth of bacteria. *Mycobacterium tuberculosis* (Mtb) secretes a tyrosine phosphatase called PtpA, essential for Mtb growth in vivo. The Mtb PtpA regulates growth by three critical mechanisms: (1) Dephosphorylation of a host protein called VPS33B and macrophage V-ATPase, which in turn prevents the phagosome-lysosome fusion and acidification of phagosomes. (2) By interacting with ubiquitin leading to repressed innate immune response. (3) By suppressing activation of NF-κB and at last by regulating transcription of various immune-related genes (Wang et al. 2017).

All viruses and some fungi also undergo intracellular replicative mechanisms. The viral genome acts as an instruction manual for synthesis of viral protein by host protein machinery. The proliferation depends on host factors and type of viral genome. Hence, the location of host factors dictates the viral proliferation; while, type of viral genome determines the infection strategy (Jaiswal et al. 2020). Different infection strategies utilized by viruses are prolonged cell cycle stages, interaction with nuclear material, and control over apoptosis and necrosis (Dyer et al. 2007). Similar to bacteria, proliferation of fungus in the human body may take place extracellularly or intracellularly. Extracellular growth of fungi takes place via attachment of fungal cells in its hyphal or biofilm form while fungi such as

Cryptococcus and some species of *Candida* germinate inside macrophage (Gilbert et al. 2015).

5.2.3.2 Microbial Biofilms

Biofilm formation is a strategy adopted by both bacteria and fungus (Vestby et al. 2020; Pappas et al. 2018). Bacterial and fungal biofilms are commonly associated with chronic diseases such as pulmonary and skin infections. Since the cellular physiology of biofilms differs from that of planktonic cells, their eradication from the human body is difficult. Pathogens strategically implement biofilm formation as a method to evade antimicrobials and escape host defense systems. Biofilm is an assembly of microorganisms enclosed in a self-protective matrix (Penesyan et al. 2020). The self-protective matrix or extracellular polymeric substance (EPS) matrix is made up of polysaccharides, nucleic acid, and proteins. The EPS matrix is able to interact and adhere to the extracellular matrix (ECM) of host tissues and also camouflages the microbial population from detection (Crosby et al. 2016). Many bacteria and fungi such as *Listeria*, *Shigella* (Bi et al. 2021), *Candida*, and molds (Costa-Orlandi et al. 2017) are known to exist in the form of biofilm. The transition from planktonic to sessile form of growth is incentivized, as the biofilms provide protection from unfavorable host defense, nutrient-rich area for colonization, and cooperative benefits of surviving as a community (Jefferson 2004). Studies have shown that the biofilm formation not only contributes to surface attachment but also a "*nonsurface-attached*" clumping as in the case of *Staphylococcus aureus* and several fungal pathogens (Kernien et al. 2018). *Staphylococcus aureus* produces several fibrinogen-binding proteins which enables coagulation of blood and bacterial aggregation. The *S. aureus* cells are held together by fibrinogen and shields the bacterial population from the host immune system (Crosby et al. 2016). Similarly, *Aspergillus fumigatus*, a fungal pathogen which causes aspergilloma, is present in tuberculosis-associated parenchymal cavities or paranasal cavities as an agglutinated spherical mass (Loussert et al. 2010). Apart from the abovementioned characteristics, several pathogens can attach to each other and form multispecies or polymicrobial biofilms. Polymicrobial biofilms have been reported in several cases of pulmonary and genital tract infections (Pekmezovic et al. 2019). One such species is *Gardnerella vaginalis*, which is able to produce polymicrobial biofilms with other vaginosis-associated species (Jung et al. 2017). Other than microbial growth, biofilms are essential for pathogen survival too, a detailed account of biofilms and its role in host–pathogen interaction will be discussed in further sections.

5.2.4 Immune Responses Against Infection

The host immune system triggers a complex array of responses to maintain the homeostasis as well as eliminate pathogenic microorganisms and allergens. The activity of foreign agents is under constant surveillance of the immune system and systematically eliminated from the body with no or minimal damage to self-tissues.

Due to the presence of a diverse number of pathogens in the environment, the eradication of microbes takes place through several broad as well as detailed selection mechanisms. Together with physical barriers, all immune responses can be categorized into two classes namely, innate immune responses and adaptive immune responses. The innate immunity is the first line of defense mechanisms and acts rapidly on pathogens by recognition of biomolecules (Turvey and Broide 2010). While adaptive immunity acts upon specific microbes, toxins, or allergens. During adaptive immune response, the responding cells proliferate once they encounter an antigen for an effective response. Further, the adaptive response manifests immune memory, i.e., it generates cells which are long-lived and can remain in dormant state for a long time so that any recurrence of infection is eliminated with an effective and much more sensitized feedback. These immune effector mechanisms exhibit self-tolerance while acting upon microbial cells. However, in case of viruses, intracellular bacteria and intracellular parasites, the immune system is obliged to destroy self-tissues which sometimes leads to a severe autoimmune response and damage to the host body. In order to distinguish between infected and healthy cells, various immune response mechanisms have evolved. One of the important mechanisms is the T-cell arm of the immune response. The T-cell acts upon infected cells of host tissue by recognizing and differentiating between foreign and self-antigens present in the lipid bilayer of cells (Chaplin 2010).

5.2.4.1 Features of Host Immune Response

The immune responses can be divided into two categories, (1) innate immune response, and (2) adaptive immune response (Turvey and Broide 2010). The innate immune response is the primary impediment implemented during invasion of foreign particles. The innate response is hardwired with germline-encoded receptors and further comprises physical and physiological barriers. The physical barriers such as tight cell-cell contacts between epithelium cells, mucus layers of respiratory, gastrointestinal, and urinary tracts behave as a blockade for contaminated particles. The physiological member of innate immunity comprises a variety of humoral and cellular components. The cellular component of innate immunity consists of leukocytes such as NK cells, basophils, eosinophils, and neutrophils. While the humoral components are small proteins and bioactive molecules which mediate inflammation and help in navigation of leukocytes to the site of infection. The complement system, defensins, cytokines, chemokines and several enzymes are the humoral component of innate immune response (Medzhitov and Janeway 2000a). These elements are ubiquitous in the host body and their concentration varies as per disease pathologies. In contrast to innate response, adaptive immune responses are manifested on the basis of sensitivity and specificity. The B- and T-lymphocytes engage in adaptive immune response. These cells have antigen-specific receptors called immunoglobulin and T-cell receptors (TCR) respectively, expressed on their surface. The lymphocyte's antigen receptors are encoded by somatic rearrangement of certain germline genes, and they provide the idiosyncratic specificities and clonal expansion of B- and T-lymphocytes. Although both immune responses have distinct characteristics and roles, they usually act together. For

example, chemokines are involved in leukocyte trafficking of several leukocytes at the site of inflammation. Various immune responses involved in host–pathogen interactions are sectioned below.

5.2.4.2 Host–Pathogen Interactions (HPIs) Involving Innate Immune Responses

As discussed earlier, innate immunity consists of physical and physiological elements. The anatomical barriers such as mucociliary clearance and enzymes present in secretions from lacrimal, salivary, and sweat glands are crucial blockades against microbes. Conditions such as primary ciliary dyskinesia have demonstrated that the sensitivity to pathogenic infections relies on the integrity of the physical barriers (Turvey and Broide 2010). The secretions such as tears, saliva, sweat, and mucus are known for several enzymes and glycoproteins, which participate in clearance of foreign agents at the entry level. Similar to these glands, the respiratory, gastrointestinal, and urogenital epithelial layers secrete mucus which protect from pathogenic bacteria by the phenomena of mucociliary clearance (Zanin et al. 2016). The mucus contains several glycoproteins such as mucins that exhibit high affinity toward bacterial recognition and are targeted for diagnostics and vaccines together with mucin-like pathogenic molecules (Pinzón Martín et al. 2019).

The physiological or humoral elements of innate immunity comprises several leukocytes and biomolecules, respectively. The leukocyte germline expresses specific receptors called pattern recognition receptors (PRRs) are able to recognize certain microbial structures known as pathogen-associated molecular patterns (PAMPs) (Kawai and Akira 2011). The PRRs is a broad term and defines all the membrane proteins present on leukocyte surface that partake in recognition of pathogens. PRRs can further be divided into three broad categories on the basis of their functional aspects. Other than PRRs, several other proteins such as complements, ficolins, defensins, and cytokines function as effectors of innate immune response. The pathogenic interaction between some innate immunity effectors and two important PRR families (TLRs and NLRs) is illustrated as follows.

Toll-like receptors (TLRs) initiate the identification of nonself-elements by recognition of multiple binding microbial epitopes. As soon as the TLRs recognize an epitope it induces a cascade of signaling response that further initiates effectors of adaptive immunity. The recognition process is sometimes catalyzed by several soluble proteins called TLR accessory molecules such as MD-2, PRATA4, CD14, and antimicrobial peptide LL37 (Jiménez-Dalmaroni et al. 2016). TLRs are homo-/heterodimeric proteins embedded in the leukocyte's cellular or endosomal membrane. The ectodomain of monomeric TLRs is composed of leucine-rich repeat (LRRs) motifs while the cytosolic part is called Toll/IL-1 receptor-like domain (TIR) that plays a significant role in signaling. The TLRs interact with flagellin, lipoproteins, nucleic acids, and other microbial ligands (Medzhitov and Janeway 2000b; Takeuchi and Akira 2010). For example, the lipoproteins, diacyl/triacyl lipopeptides, porins, and other soluble factors present in bacteria are recognized by TLR1, TLR2, TLR5, and TLR6 (Takeda et al. 2003; Lee et al. 2012). Similarly, envelope and fusion proteins of viruses (Zhou et al. 2021) and fungal proteins such

as zymosan are recognized by TLR4 and TLR2 respectively. While TLR3 and TLR9 specifically bind to ds-DNA and CpG DNA of viruses and bacteria (Takeda et al. 2003; Takeda and Akira 2015). Two kinds of signaling cascades namely, MyD88/MAL and TRAF/TRAM pathways are activated upon binding of microbial ligands to TLRs. For instance, TLR2/TLR6 or TLR2/TLR1 receptor dimers activate a downstream MyD88/MAL pathway, while TLR4 homodimer can stimulate either MyD88/MAL or TRAF/TRAM signal pathway (Hill and Diehl 2018; Price et al. 2018). Upon successful signal transduction, MyD88/MAL expressed transcription factors AP-1, NF-κB initiates production of inflammatory cytokines; while TRAF/TRAM expresses IRF transcription factors for transcription of type I interferon (Zhou et al. 2021) (Fig. 5.1). Several mutations in TLRs and adaptor molecules lead to increased susceptibility toward microbial infections. For instance, mutation in ligand-binding regions of TLR5 and TLR1 adaptor molecules—MAL/TIRAP makes the host system more susceptible to legionnaire, malaria, mycobacterial, and pneumococcal infections (Turvey and Broide 2010).

NOD-like receptors (NLRs) are a family of receptors that inspect the intracellular milieu and sense microbial flagellin and metabolic stress. The human NLRs are a family of 28 members. Upon detection of microbial activity, the NLRs trigger formation of inflammasomes that further activate inflammatory caspases and expression of IL-1β and IL-18 cytokines (Sharma and de Alba 2021). The NOD (Nucleotide-binding oligomerization domain) proteins or NLRCs, IL-1β-converting enzyme protease-activating factor (IPAF) are able to detect bacterial peptidoglycans and flagellin (Carriere et al. 2021). Another NLR family, NLRPs detect intracellular metabolite-related stress. NLRP3 or cryopyrin is activated by inflammation-mediated potassium efflux, uric acid, and increased extracellular ATP. Similar to TLRs, mutations in NLR proteins are associated with chronic diseases in which normal host microflora is involved. An ideal illustration of the same has been noted in case of patients suffering with Crohn disease. The patients suffering from Crohn disease carry NOD2 mutations in at least one allele in the leucine-rich repeat causing partial truncation of LRRs. In cases where NOD2 mutations are present, the mutant NOD2 exhibits no affinity toward muramyl dipeptide, a bacterial peptidoglycan component and triggers gut microflora-associated inflammation by deficient expression of IL-10. Examples of inflammation caused by NLR-associated mutation and gut microflora involving NLRP6 has also been reported (Turvey and Broide 2010; Guo et al. 2020). The structures and microbial ligands of some NLRs are illustrated in Fig. 5.2.

Other important PRRs that contribute to identification of microbes include **dectin-1 (C-type lectin receptor family), collectins, ficolins**, and **pentraxins**. Dectins are transmembrane proteins that recognize β-glucans of fungal cell wall (Saijo and Iwakura 2011). The collectins and ficolins are a group of proteins, which recognize microbial carbohydrates in various respiratory syndromes, and produce reactive oxygen species (ROS) (Heesterbeek et al. 2018). While pentraxins are homopentameric soluble proteins which bind to bacterial low-density lipoproteins, polysaccharides and induce activation of the complement system. Two best

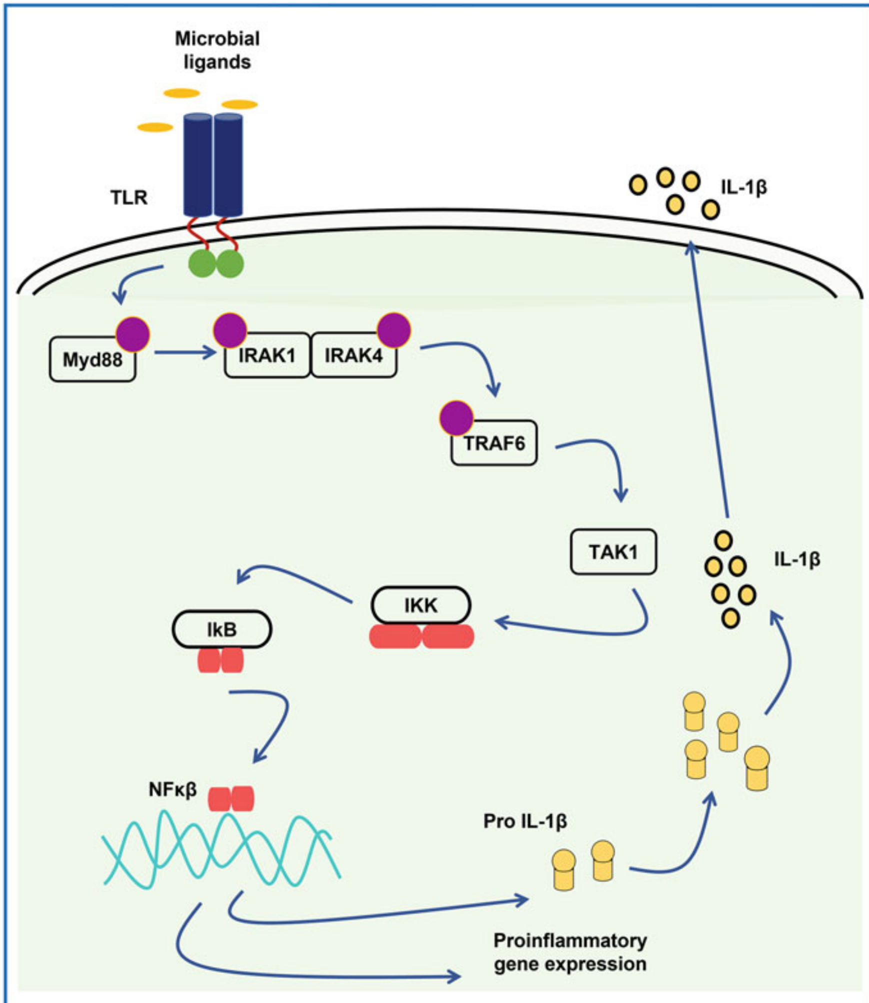

Fig. 5.1 Schematic illustration of the TLR pathway activated upon their interaction with microbial ligands resulting in expression of proinflammatory cytokines and NF-κB

examples of pentraxins are C-reactive protein (CRPs) and serum amyloid P-component (Chaplin 2010).

Several other soluble effectors of innate immune response are chitinase, complement system, defensins, and cytokines. **Chitinases** are a family of proteins that target chitin, a major constituent of fungi and helminths. Human chitinases such as YKL-40 protein, an acidic mammalian chitinase binds and sequesters chitin, which upon degradation exhibits immunomodulatory activity (Chaplin 2010). The **complement system** is a group of plasma and cell-surface proteins that regulates regulatory signal transduction via three different pathways. Most of the complement

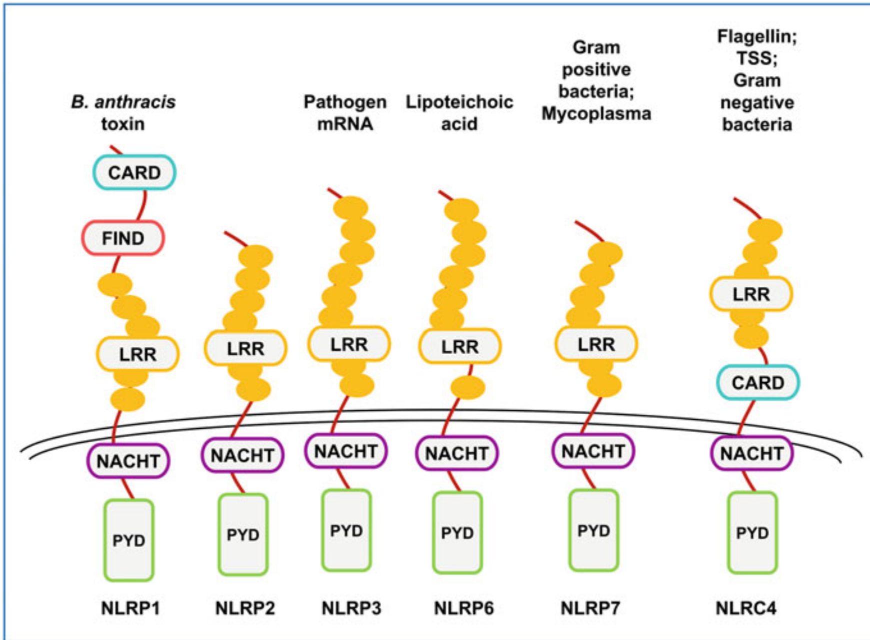

Fig. 5.2 Schematic representation of various types of NLRs and their interacting microbial ligands

system proteins are proteinases that activate a cascade of zymogen to recruit proteins involved in osmotic lysis of viruses and bacteria. The complement system is also involved in opsonization, and macrophage-mediated phagocytosis. In several cases, deficiencies of components of certain complements lead to elevated susceptibility to infections. For instance, deficiency of components of complement membrane attack complex leads to increased sensitivity toward *Neisseria*-led infections (Medina 2016). **Defensins** and **cathelicidins** are small antimicrobial peptides involved in bacterial and fungal infections (Yang et al. 2001).

Another family of effectors of innate immunity is **cytokines**, which mediate inflammation, leukocyte trafficking, and phagocytosis. Cytokines are small soluble proteins which act on leukocytes via GPCRs-mediated signaling pathways such as JAK-STAT pathway (Thaiss et al. 2014; Tripathi and Poluri 2020). The cytokines consist of several protein subfamilies such as interferons and chemokines which are involved in several infections. **Interferons** (INF) are a group of cytokines having antiviral effects. They are classified into three different classes type I, II, and III. Several INF-induced proteins such as tetherin, viperin, APOBEC3G (cytidine deaminases), and protein kinase R are significantly involved in inhibition of viral replication, reverse transcription, and translation (Biron 2016). The cytokines and other effectors of innate response also act as effectors and mediators of adaptive immune response. Several other bacteria also utilize glycoproteins like **mucin** and **lectins** for initiation and promotion of infections. For example, Lewis B glycosylated

mucin proteins are vital for host binding and invasion in case of *Helicobacter pylori* (Xu et al. 2020). The host–pathogen interactions involving mucins are discussed in detail in later sections.

5.2.4.3 HPIs Involved in Adaptive Immune Response

Unlike innate immunity, adaptive immune response is precisely tuned for targeting nonself elements. The **antigen-presenting cells (APCs)** together with **T** and **B lymphocytes** mediate immunologic response and memory. The diverse repertoire of receptors present on lymphocytes is able to identify principal features of potential foreign agents. Similar to innate immunity, adaptive immune response is mediated via cellular as well as humoral components. The cellular response takes place with the help of T-cell which matures in thymus; while humoral response, i.e., antibody production is mediated by B-cells, which are produced in bone marrow. The interleukins such as IL-7 play a significant role in expression of T and B lymphocytes (Bonilla and Oettgen 2010). The APCs are a group of immune cells which bind to antigenic peptides and present them to T-cells with the help of **major histocompatibility complex (MHC)** molecules. Two different classes of MHCs, MHC I and MHC II are expressed by APCs for activation of T-cells. Upon activation, the helper T-cells (Th-cells) initiate expression of inflammatory cytokines, while the natural killer T-cells (NKT cells) and cytolytic T-cells (CTLs) phagocytose pathogens and infected host cells respectively. Other than phagocytosis, T-cells stimulate development and clonal selection of B-cells from their progenitor plasma cells. The B-cells contain immunoglobulin-encoding genes. The **immunoglobulins (Igs)** are high-molecular weight protein molecules which participate in antigen–antibody interactions. The immunoglobulin is a family of five members, IgM, IgG, IgA, IgD, and IgE. The Ig molecules are tetrameric proteins containing two heavy and two light chains bound by disulfide bonds. These Igs recognize microbial epitopes and stimulate antibody-mediated cellular cytotoxicity (ADCC) or complement-mediated cytotoxicity (Schroeder and Cavacini 2010).

5.2.4.4 Immune Response Via Metabolite Sequestration

The immune response functions on a vast front and induces immune response from genetic to cellular level. In retaliation to microbial invasion, the host immune system triggers several gene pathways, autophagy, and regulation of essential nutrients such as iron (Monteith and Skaar 2021). The initial response to invasion activities is activation of numerous immune signaling cascades. The activation and expression of immune-related genes involved in these cascades particularly depend on the type of pathogen and their pathogenicity. The feedback action against bacterial species mostly involves protein–protein interactions, and the involved biomolecules are in constant mode of evolution to maximize their biological role and adapt to the evolutionary arms race. Similar to pathogens, the host immune system also modulates process such as metabolite sequestration, cellular trafficking, and the cytoskeletal system as defensive mechanisms during infections (Schweppe et al. 2015). One of the most magnificent yet simple mechanisms is sequestration of crucial metabolites, as metabolite assimilation during infection promotes microbial

population and gene expression (Monteith and Skaar 2021). Several bacterial proteins show high affinity to host proteins involved in nutrient transport. The bacterial proteins direct the nutrient transport toward the bacterial cell rather than the host cell. For instance, iron (Fe) being an important element in the respiratory chain, its uptake and sequestration are affected for both host and the bacteria (Nairz and Weiss 2020). It is also a major regulator of cell cycle, i.e., in absence of Fe, the cyclin and cyclin-dependent kinase formation and activity are inhibited. In the human body, Fe is predominant in a bound state with several proteins such as, hemoglobin, myoglobin, transferrin, and lactoferrin, resulting in low concentrations of free Fe in the bloodstream. The free Fe concentration in blood is about 11–32 μmol/L, and is inadequate for the bacterial growth. Hence, in order to obtain Fe, pathogens contain iron-chelating proteins such as transferrin-binding protein, TbpA (Bullen et al. 1978; Zughaier and Cornelis 2018).

5.3 PPIs in Bacterial Infections

Bacteria are prokaryotic organisms that are ubiquitous and of immense importance ecologically. They carry genetic information through classical-stranded DNA and also with small circular plasmid. Many bacterial species have been a significant part of the human microbiota unique to their ecological conditions. Of these, only a subpopulation is known to cause infection in humans. The cytoplasm is enclosed in a cell membrane and cell wall (except *Mycoplasma*) and contains ribosomes without any compartmentalization. Their movement depends on the presence of cilia, flagella, or pili, and it reproduces asexually. Based on the composition of the cell wall, it is divided into two groups: Gram-positive and Gram-negative bacteria (Persat et al. 2015). Both classes of bacteria are reported to cause several acute diseases like Tuberculosis, Cholera, and Psoriasis. They are also involved with chronic diseases such as Inflammatory Bowel Syndrome (IBS), Asthma, and immunosuppressive disorders. These are transmitted via reservoirs like water, food, soil, or a vector (Doron and Gorbach 2008). Other than opportunistic pathogens, the nonpathogenic commensal strains can also often cause a state of disease. The pathogenesis primarily depends on expression and action of virulence factors. The type of virulence factors, quorum sensing (QS) pathways, and morphological features are defining factors of host–pathogen interactions involved in important bacterial diseases. This section enunciates variety of bacterial virulence factors, and their mode of action involved in host–pathogen interaction in a detailed manner.

5.3.1 Bacterial Virulence Factors

Bacterial virulence factors are produced in order to assist bacteria for host invasion, infection, and evasion of host defenses. They often include signal peptides/transcription factors that modulate the downstream translation/transcription cascade in order

to bring about compromised native cellular physiology. These virulence factors modify the gene-expression/vicinal environment such that the pathogenic proliferation is favored. These factors are also believed to mimic the host biomolecules and trigger anomalous cellular pathways. Diverse types of virulence factors such as adhesion factors, bacterial secretion pathways, invasive protein, and exotoxins are described below:

5.3.1.1 Adhesion and Invasion Factors

Adhesion/invasion factors start the process of bacterial colonization on biological and inert surfaces. Later, they mediate biological invasion, biofilm formation, and survival of pathogens on various surfaces. Adhesion to biological surfaces takes place by specific protein–protein and other biomolecular interactions between the host and bacterial pathogen. The adhesion factors predominantly target cellular receptors, cell-surface glycoproteins, and cytoskeletal proteins. These factors are collectively known as **adhesins** (Kline et al. 2009). Adhesins are expressed on cell surfaces of bacteria and have affinity toward extracellular matrix and transmembrane proteins such as collagen, elastin, integrins, and cadherins (van Belkum et al. 2021). Microbial adhesins interact with a variety of host transmembrane receptors based upon their origin. For instance, viral adhesins associate with both glycan-based and protein receptors and differ in their structures as has been deciphered in case of SARS-CoV-2 variants (Peacock et al. 2021). Similarly, the structural composition of adhesins varies between Gram-negative and Gram-positive bacteria as well. In Gram-negative bacteria, adhesins can be divided into fimbrial (pili), nonfimbrial, and polysaccharide adhesins. On the other hand, other than fimbrial and nonfimbrial adhesins, Gram-positive bacteria express "Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) on their cellular surfaces (Vengadesan and Narayana 2011).

Fimbrial Adhesins

The **Fimbrial adhesins or pili** are oligomeric fibers of pilin protein. In general, pili are associated with motility and attachment. However, they also play a role in immunomodulation, DNA transfer, and antigen presentation. Two types of pili that are majorly responsible for virulence in many bacterial pathogens are: **type I fimbriae** (T1F), and **type IV pili** (T4P). The type I fimbriae participate in initial stages of biofilm formation. The classic example of the role of type I fimbriae in infections is accumulation of Uropathogenic *E. coli* (UPEC) communities during chronic UTIs (Müller et al. 2009). The *E. coli* T1F adheres to the mannosylated urothelial receptors such as uroplakin 1a (Zhou et al. 2001), and desmoglein2 (McLellan et al. 2021) promoting formation of *E. coli* colonies. The T1F adhesins are expressed by *fim* operon, which expresses recombinases (FimE/B), structural components (FimA/F/G/H), and pilus assembly (FimC/D). The structural component FimH is mainly responsible in receptor interactions and inhibition, and no expression of FimH leads to reduced adhesion of UPEC (Avalos Vizcarra et al. 2016). Another fimbrial adhesin called type IV pili (T4P) is expressed in several groups of Gram-negative bacteria and Gram-positive bacteria. The T4P enables the

Table 5.1 Types of fimibrial adhesins/pili and their target molecules in host

Type of pili	Host receptor	Example of species
Type I pili	Glycoproteins and glycolipids	*E. coli*
Type IV pili	Glycolipids, C4BP	*E. coli*, *Neisseria*, *Pseudomonas*
Curli pili	Fibronectin, laminin, plasminogen	*E. coli*
Fibrils	Fibronectin	*S. salivarius*
Pili	Collagen	*Streptococcus*, *Corynebacterium*

bacteria to move using the twitching motility, i.e., the ability to retract through the bacterial cell wall, while the pili surface remains attached to the host tissue. *Neisseria meningitidis* utilizes the T4P system to colonize, proliferate, and remain aggregated in vascular endothelial cells (Ribet and Cossart 2015). T4P present in Gram-negative bacteria is broadly classified into two classes namely, type IVa and type IVb. On the other hand, Gram-positive bacteria express two kinds of pili namely, Type IV-like pili, and sortase-associated type IV-like pili (Telford et al. 2006; Danne and Dramsi 2012). A brief classification of other fimbrial adhesins are mentioned in Table 5.1.

Bacterial Secretion Systems

Bacterial pathogens also utilize **secretion systems** for invasion into the host cells (Jaiswal et al. 2019). The secretion systems are specialized protein assemblies which inject "effector proteins" into the host cell once they attach to it (Southwood and Ranganathan 2019). Originally, eight classes of protein secretion systems namely Type I, Type II, Type III, Type IV, Type V, Type VI, ESX or Type VII, Sec/Tat secretion systems were identified. Based on their composition, such secretion systems are found either in Gram-negative, Gram-positive, and or *Mycobacterium* (Costa et al. 2015). The secretion systems comprise of channel proteins embedded in phospholipid membranes and motor proteins such as pili and flagella. They stick/ adhere to the host cell membrane and ensure a proper channel for secretion of toxins in the form of nucleic acid, protein, or other biomolecules. Each of these secretion systems differs structurally and functionally (Green and Mecsas 2016). For example, Type I secretion system (T1SS) which is found predominantly in Gram-negative bacteria, has a "barrel"-like structure. It is made up of three components namely, an ABC transporter protein, a membrane fusion protein, and the outer membrane factor. It transports substrates such as adhesins, heme-binding proteins, and proteins with repeat-in-toxin (RTX) motifs (Green and Mecsas 2016). T1SS is utilized by several bacterial pathogens such as *V. cholerae* and *E. coli* to secrete toxins like MARTX and HlyA (Spitz et al. 2019). Whereas, another ubiquitously found secretion system of Gram-negative bacteria called Type III secretion system (T3SS) is a syringe-like apparatus that secretes only protein substrates into the extracellular milieu or directly across host cell membrane (Hotinger et al. 2021). T3SS has been identified to be involved in secretion signaling in various pathogens like *Pseudomonas*, *Shigella*, enterohemorrhagic *E. coli* (Green and Mecsas 2016). The secretion system also varies depending on the type of bacterial cell membrane morphology owing to this, Gram-positive bacteria including *Mycobacterium*, have distinct protein secretion

systems for increasing the efficiency of bacterial invasion. *Mycobacteria* contain a cell wall layer known as mycomembrane, which is lipid-rich and extremely hydrophobic in nature. The presence of mycomembrane provides a survival advantage to *Mycobacterium;* however, it makes the transport of extracellular proteins difficult. To overcome this, the *Mycobacterium* strains and several Gram-positive bacteria have a special secretion system called Type VII secretion system (T7SS) of ESX system (Green and Mecsas 2016). The structural components of the T7SS secretion system are membrane proteins and differ in bacterial species based upon structure of bacterial cell membrane.

Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Surface proteins also function as adhesive molecules especially in case of gram-positive bacteria. The surface proteins involved in adhesion are known as "**Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs**). MSCRAMMs are involved in bacterial adhesion to the ECM proteins such as collagen (Co), fibrinogen (Fb), laminin (La), and fibronectin (Fn). The structure of MSCRAMMS is composed of an N-terminal sequence for secretion across the bacterial membrane, mid binding region, and C-terminal transmembrane region with a sortase recognizable "LPXTG" motif. The MSCRAMMs interaction with ECM proteins has been explained by three models namely (1) the collagen hug model, (2) dock, lock, and latch model, and (3) tandem β-zipper model (Foster 2019). The MSCRAMMs are generally expressed by Gram-positive bacteria except fibronectin-binding proteins (FnBPs). The FnBPs are expressed by both Gram-positive and Gram-negative pathogens. Further, a brief description to three sub-classes of MSCRAMMs is provided:

Collagen-Binding Proteins (CoBPs)

Collagen (Co) is the ubiquitous skeletal protein present in vertebrates. It is one of the major components of the extracellular matrix (ECM). Several MSCRAMMs adhere to collagen for internalization as well as biofilm formation. A well-studied example of CoBPs is CNA (Collagen-binding adhesin) expressed by *S. aureus* (Holderbaum et al. 1986; Vengadesan and Narayana 2011). The CNA assists the pathogenesis of *S. aureus* infections such as osteomyelitis, endocarditis, keratitis, and arthritis (Madani et al. 2017). Xu et al., explored the virulence of CNA adhesin and found that CNA's affinity toward collagen drives the virulence of *S. aureus*-mediated septic arthritis in mice models. The mice were infected with *S. aureus* and isogenic forms of *S. aureus* expressing inactive CNA and progression of infection was observed 24-h postinoculation. A significant decrease in *S. aureus* infections was observed in case of mutant CNA (Xu et al. 2004). The formation of CNA-collagen complex involves polar and hydrophobic interactions, and has been explained by the "collagen-hug model" (Fig. 5.3) (Liu et al. 2007). CNA protein comprises of three domains: (1) N-terminal domain containing three subdomains (N1, N2, and N3) which interacts with collagen, (2) a linker region which links the N1 and N2 subdomains, and (3) C-terminal cell wall/membrane-spanning domain (Foster

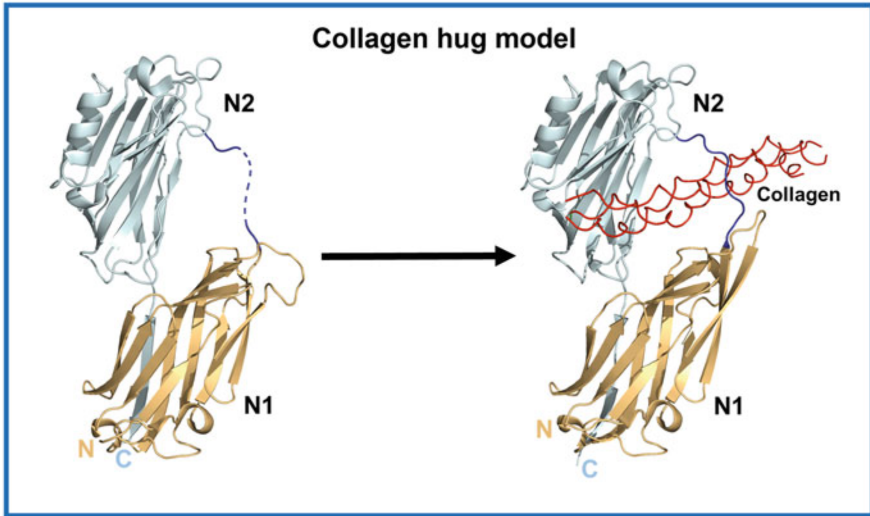

Fig. 5.3 CNA–collagen interaction through "Collagen hug model." In apo form (left; PDB ID: 2F68), the N1 (golden) and N2 domain (light blue) are linked by a linker region (dark blue) which is the binding hotspot of collagen. In presence of collagen (red), the linker region interacts and wraps around collagen forming the CNA-collagen complex (right; PDB ID: 2F6A)

2019). The N1N2 subdomains wrap around the collagen triple helix and are the exclusive hotspots for collagen interaction. Moreover, the interaction between CNA and collagen is a two-step mechanism, where initially the N2 subdomain interacts with the collagen that induces structural rearrangements in the N1 subdomain, thus repositioning it closer to the N2 subdomain. The repositioning forms a tunnel-like structure that enables insertion of C-terminal end of N2 into N1 subdomain and forming a latch around collagen (Arora et al. 2021). *Streptococcus mutans*, *Enterococcus faecalis*, and *Enterococcus faecium* also express homologous collagen adhesins/CoBPs called CNM (collagen and laminin-binding protein), ACE, and ACM, respectively, which are associated with infections of the cardiovascular system such as hemorrhagic stroke and endocarditis (Madani et al. 2017).

Fibrinogen-Binding Proteins (FgBPs)

Fibrinogen (Fg) is a skeletal protein found in blood plasma that maintains the homeostasis and mediates the coagulation process. Pathogens such as *S. aureus* utilize Fg to form "nonsurface attached biofilms." Fg is a hexamer made up of three homodimers ($\alpha_2\beta_2\gamma_2$). The S. aureus adhesins—clumping factor A (ClfA) and clumping factor B (ClfB) target the γ-chain and α-chain of fibrinogen, respectively resulting in Fg-mediated cell clumping. The affinity of ClfA is inversely dependent on the presence of divalent cations such as Ca^{2+} and Mn^{2+}. Another species of *Staphylococcus*, *S. epidermidis* expresses SdrG adhesins that are similar to ClfA and ClfB, and shows affinity toward β-chain of fibrinogen (Madani et al. 2017). Studies have shown that colonization of *S. aureus* is regulated by low temperature and pH in

epithelial tissues. The lower pH triggers digestion of filaggrin, an epidermal filament-aggregating protein, into urocanic acid and pyrrolidone carboxylic acid. The digested products have been shown to downregulate the expression of bacterial expression factors such as clumping factor B (ClfB) and fibronectin-binding protein A (FnbpA), important for colonization of *S. aureus* (Krishna and Miller 2012). The clumping factors (Clf) share a similar 3D structure as the CoBPs, where the N2 and N3 domains of the Clf are majorly involved in the interaction with fibrinogen (Ganesh et al. 2008). A recent study identified allantodapsone as a pan-inhibitor of the Clf–fibrinogen interaction that can be used to inhibit nasal and skin colonization of *S. aureus* in wound infections (Prencipe et al. 2022).

Fibronectin-Binding Proteins (FnBPs)

Fibronectin (Fn) is a glycoprotein involved in cellular processes such as cell adhesion, growth, tissue repair, and blood coagulation. It is found in the ECM matrix and blood plasma. Fn interacts with transmembrane integrins and is able to interact with collagen and laminin. Fibronectin is commonly targeted by pathogens causing gastrointestinal infections. The FnBPs target Fn with the help of specific-binding repeats (FnBRs) present in the C-terminal region (Moschioni et al. 2010). The two extensively studied staphylococcal FnBPs are FnBPA and FnBPB. Both FnBPs are anchored to the cell wall by LPXTG motif, while the fibronectin-binding site has 11 and 10 tandem repeats of 30–40 amino acids in FnBPA and FnBPB respectively (Henderson et al. 2011). The FnBPA/B exhibits multivalent binding, and the FnBR's tandem repeats interact with the N-terminal region of Fn by a tandem β-zipper model. Several adhesins have also been identified which do not contain the LPXTG motif or generic MSCRAMMs structure but show affinity toward fibronectin. For instance, Ebh (Extracellular matrix-binding homolog) and Emp (extracellular matrix protein) are cell wall-embedded protein in *S. aureus* that lacks LPXTG motif but contains mid region repeat sequence for interaction with Fn (Josse et al. 2017). Ebh plays a major role in determining the cell growth and shape, while Emp shows broad affinity toward ECM components such as collagen, fibrinogen, and vitronectin other than Fn (Hymes and Klaenhammer 2016). *S. aureus* expresses a wide variety of FnBPs targeting a variety of host receptors (Fig. 5.4); although, FnBPs vary genus to genus. The Streptococcal species, *S. pyogenes*, expresses approximately 11 different FnBPs. Two of the important streptococcal FnBPs are F1 and Sfb1. Similar to FnBPA/B, F1 and Sfb1 are cell walls anchored and contain the central FnBR region. Streptococcal FnBPs that share FnBR sequence similarity with *S. aureus* are F2, FbaB, Sof, SfbX, and FbaA. *S. pyogenes* expresses a second subset of FnBPs which do not exhibit canonical-binding repeats. These include M1 protein, Shr, and Scl1 (Hymes and Klaenhammer 2016; Josse et al. 2017).

Previously, it has been assumed that the MSCRAMMs/adhesins exhibit single-ligand specificity. However, in recent years a multifunctional model for molecular mechanics behind bacterial adhesins has been emerging (Speziale and Pietrocola 2020). The theory behind a multifunctional model comes from the fact that there are a limited number of cell wall-associated proteins expressed by a cell, which must perform multiple functions other than adhesion (such as invasion, evasion, and

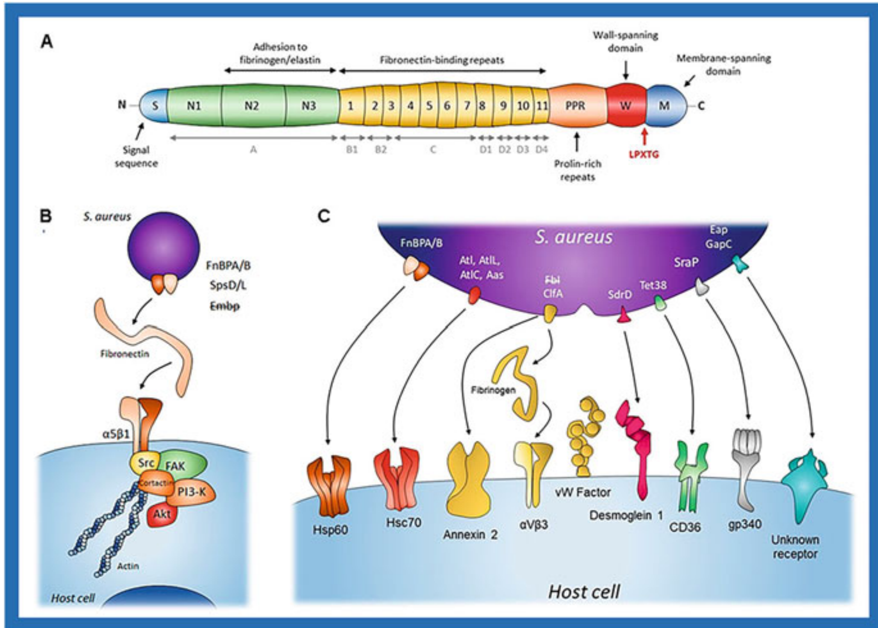

Fig. 5.4 (**a**) Schematic of *S. aureus* FnBP. The keywords in gray represent the alternative nomenclature of FnBP repeats. (**b**) Depicting the interaction of FnBP with fibronectin-integrin complex for internalization of *S. aureus* cells. (**c**) Glimpse of the targeted host proteins by various *S. aureus* FnBPs. (Adapted from Josse et al. 2017)

biofilm formation). Hence, adhesins have evolved to exhibit affinity toward multiple ligands. Enzymes such as glyceraldehyde-3-phosphate-dehydrogenase (GaPDH), peptidases (Ssa in *S. suis*), and endopeptidases (PepO in *S. pneumoniae*) have also been identified as fibronectin-binding proteins.

Capsule and Other Surface Components

A bacterial cell undergoes a variety of stress upon gaining entry inside the host. To overcome this stress and enhance their survival rate, many bacteria utilize capsule formation as a protecting mechanism. The capsules are generally made up of proteins, lipids, and carbohydrates. It camouflages the bacterial cells from phagocytic cells and elevates the virulence of bacteria (Hyams et al. 2010). In pneumococcal infections, it was evident that encapsulated strains are more virulent and resistant compared to the nonencapsulated strains. The capsule layer in several bacteria contains antigens that mimic the host proteins and lipopolysaccharides. The camouflage property helps in evading host immune response. Moreover, because of high selective pressure, the capsular antigens evolve at a higher rate than other virulence factors. For instance, *Salmonella typhi* carries *Vi* antigen which undergoes constant mutations resulting in increased virulence and novel strains. *Vi* antigen is a polysaccharide composed of galactosamine and uronic acid linked by 1,4-linkage (Johnson

et al. 2018). Several strains of *B. cereus* are enclosed in a surface glycoprotein layer (S-layer) which mediates locomotion, toxin production, and adhesion, respectively. The S-layer proteins (SLP) also provide resistance to γ-ray radiation (Tuipulotu et al. 2021). The SLPs are exceptionally inflammogenic and are a major cause of *Bacillus*-mediated endophthalmitis. SlpA, a S-layer protein expressed by *Bacillus thuringiensis* has been characterized to contribute to pathogenesis of endophthalmitis in mice experimental model (Mursalin et al. 2019).

5.3.1.2 Bacterial Toxins

Pathogens produce diverse types of secretory molecules or toxins that help in weakening the host immune system. Bacterial toxins are of two types and called as endotoxins and exotoxins. Endotoxins are lipopolysaccharides, while exotoxins are protein toxins released from the bacteria. The following sections describe various types of toxins produced by bacterial pathogens involved in infections.

Endotoxins

Endotoxins are toxic lipopolysaccharides present in Gram-negative bacteria (Wang and Quinn 2010). These are toxic substances released after lysis of a bacterial cell. Structurally, bacterial endotoxins have a common pattern with specific species and genus diversity and have been elaborately studied in *Salmonella* spp. and *E. coli*. The structure of endotoxins comprises three regions: (1) O-specific oligosaccharide chain, (2) core polysaccharide backbone, and (3) lipid A, which consists of glucosamine disaccharide with a long chain of fatty acid and phosphate (Trent et al. 2006). The lipid A confers the toxicity, while antigenic diversity is due to the polysaccharide backbone. Toxicogenic response elicited due to endotoxins includes complement system activation, pyrogenicity, curtailed blood pressure, and several other pertinent biological responses. The potential use of artificial/truncated endotoxins as drugs and vaccines has been gaining a lot of traction and holds a promising lead for invention of safe treatment methods (Mulder et al. 2019). In an attempt to improve the antiendotoxin activity, a variant V13P (VARGWGRKCPLFG) peptide of VG16KRKP (VARGWKRKCPLFGKGG) was created, and a direct correlation between compact structure and antiendotoxin activity was established by Datta et al. through NMR-based studies (Datta et al. 2017).

Exotoxins

Exotoxins are protein toxins secreted by viable bacterial cells responsible for aggravating infections in the host body (Sakari et al. 2022). They have no noticeable effect on the bacterial growth but help in creating a sustainable environment for microbial proliferation by targeting the host immune response. Exotoxins exhibit a localized effect, i.e., on specific cell types or tissue. For example, botulinum toxin acts specifically on motor neurons (Sastalla et al. 2016). Exotoxins are categorized into three categories: (1) cytotoxins, (2) neurotoxins, and (3) enterotoxins based upon their target cell type or biological role (Cavaillon 2018).

Cytotoxins

Cytotoxin is a heterogenous group, which has a direct effect on specific cell types. Bacteria express a wide array of cytotoxins, which target cellular regulators and eukaryotic switches. Most of the cytotoxins are enzymes that regulate their action by altering structural or functional modifications in eukaryotic proteins. For instance, diphtheria toxin (*Corynebacterium diphtheriae*) inhibits protein synthesis by catalyzing ADP-ribosylation of elongation factor II, blocking the elongation of the peptide chain (Aktories and Barbieri 2005). General targets of cytotoxins are GTP-binding proteins specially GTPases belonging to the Rho family. Rho GTPases are a subfamily of Ras proteins which function as "molecular switches" in eukaryotic signal transduction pathways and regulate actin cytoskeleton arrangement. Rho proteins are regulated by the GTPase cycle. The GDP-bound state of Rho GTPase is inactive and associated with guanine nucleotide dissociation inhibitor (GDI). Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) with the help of GDP → GTP exchange. The active Rho GTPase induces downstream signal transduction and is inactivated by a GTP → GDP exchange catalyzed by GTPase-activating protein (GAP). Rho GTPases are involved in cytoskeletal rearrangement processes such as morphogenesis, migration, phagocytosis, and other cellular functions such as cell cycle progression, transcription, ROS production, and apoptosis (Boureux et al. 2007). The regulation of actin cytoskeleton by Rho GTPases is most important in case of infection. The bacterial toxins mimic eukaryotic GEFs and GAPs to modulate the downstream signaling pathway. The cytotoxins interfere with all phases of GTPases cycle. The cytotoxins perform both reversible and irreversible modification of Rho GTPases. The irreversible inhibition of Rho GTPases takes place by covalent modification such as glucosylation (clostridial toxins A and B), ADP-ribosylation (C3-like exoenzyme, diphtheria toxin), and proteolytic cleavage (YopT). While reversible inhibition takes place by bacterial effectors which mimic GAP or GEF activity for example YopE (*Yersinia enterocolitica*), SptP (*Salmonella*), and ExoS/T (*Pseudomonas*) (Fig. 5.5) (Aktories 2011; Bielek and Schmidt 2012).

Glucosylating Cytotoxins

Glucosylating cytotoxins are found exclusively in *Clostridium difficile*. *C. difficile* produces Toxins A and B which cause antibiotic-associated diarrhea and pseudomembranous colitis. The clostridial toxins are classified as AB-toxin as they contain an N-terminal enzyme domain and a C-terminal binding domain. While the central hydrophobic region mediates the translocation across the membrane. Toxin A and B glucosylate RhoA (Thr37), Rac (Thr35), and Cdc42 (Thr35) and block the subsequent signal transduction pathway through inhibition of GEF and GAP. The Rho protein stays in its inactive state, i.e., bound to GDI (Guanine nucleotide dissociation inhibitor) which causes severe redistribution of actin. Inhibition of Rho GTPases by clostridial toxin further results in inhibition of phospholipase D activity, apoptosis, chemokine-receptor signaling, and endothelial barrier function (Voth and Ballard 2005; Schmidt and Aktories 2020). Moreover, toxins A and B stimulate a massive inflammatory response by activation of proinflammatory

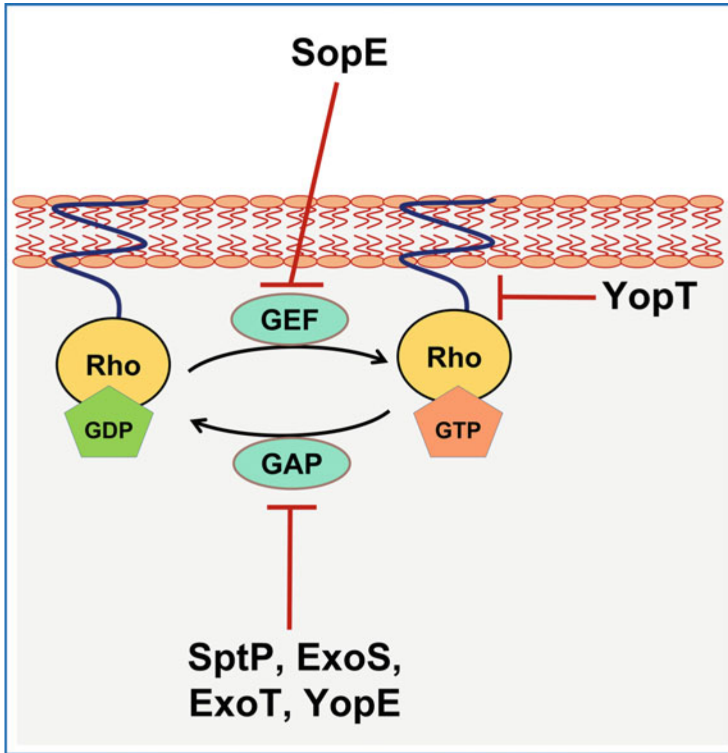

Fig. 5.5 Schematic illustration of three different approaches taken by cytotoxins for inhibition of Rho molecular switches thereby inhibiting conversion of GDP to GTP and vice versa

cytokines such as IL8 and MCP2, prostaglandins, and leukotrienes (Bielek and Schmidt 2012).

Rho-ADP-Ribosylating Cytotoxin

ADP-ribosylating cytotoxins perform ribosylation of Rho GTPases. The first identified Rho-ADP-ribosylating cytotoxin is exoenzyme C3 from *C. botulinum* (Chen and Barbieri 2018). C3 inactivates RhoA, RhoB, and RhoC through ribosylation of asparagine residue. For example, C3 ADP ribosylates RhoA at Asn41, blocks activation of Rho by GEFs, and stabilizes RhoA-GDI complex similar to the glucosylation (Aktories and Schmidt 2014). Like glucosylation, inactivation of Rho protein by ADP-ribosylation alters the morphology of the cell (Catara et al. 2019). C3 exoenzyme is expressed as a precursor containing a catalytic and an auto transporter system, mature C3 consists only of catalytic domain. C3 enzymes act upon the Rho proteins present in the cytosol. The enzyme is transported inside the cell either by pore-forming toxins such as botulinolysin and cereolysin O, or it is produced by internalized pathogens (Aktories et al. 2011).

Proteolytic Cytotoxins

Several Rho-inhibiting cytotoxins function by cleaving the Rho protein and breaking down the GTPase cycle. Yop family of proteins are enzymes involved in irreversible and reversible proteolytic cleavage of Rho GTPases. The cytotoxins belong to *Y. enterocolitica* which causes gastrointestinal infections. Yop family consists of six effectors namely, YopH, YopE, YopJ/P, YopO/YpkA, YopT, and YopM. YopT is a protease which irreversibly cleaves Rho GTPases at Cys104 at the C-terminal end and releases the post-translationally modified Cys (Ribet and Cossart 2010). The Cys is a part of the CaaX box, which plays a vital role in post-translational modification of Rho proteins. The CaaX box is the site of isoprenylation (geranylgeranylation) and carboxymethylation of Cys residue (Kamath and Kamanna 2022). The prenylated cysteine residue is essential for the membrane binding of active GTPase and proper function of switch protein. Moreover, YopT inhibits formation of GTPase-GDI complex and renders cellular function. YopT and other members of the Yop family (YopE, YopO, and YopH) aid *Y. enterocolitica* in evading phagocytosis. YopE and YopO cleave Rho protein while YopH is a tyrosine phosphatase which dephosphorylates host proteins such as focal adhesion kinase, CAS, paxillin, etc. (Roppenser et al. 2009).

GAP-Mimicking Cytotoxins

The inhibition of Rho proteins by cytotoxins is reversible in many cases. YopE (*Yersinia*), StpP (*S. enterica*), and ExoS and ExoT (*P. aeruginosa*) are bacterial GAPs which mimic the eukaryotic GAPs (Isaksson et al. 2009). These effectors are delivered by the T3SS secretion system and inactivate Rho GTPases. The N-terminal of the cytotoxins possesses the GAP activity, while the C-terminal domain of these effector toxins exhibits other enzymatic activities. For example, The C-terminal of StpP has a tyrosine phosphatase domain and the C-terminal of ExoS and ExoT possesses FAS-dependent ADP-ribosyl transferase activity. Moreover, StpP protein induces depolymerization of actin stress fibers and catalyzes the internalization process of *Salmonella* (Isaksson et al. 2009).

Neurotoxins

Neurotoxins are exotoxins that target neuronal cells affecting the nervous system and its homeostasis (Popoff and Poulain 2010). Several enterotoxins and cytotoxins affect the nervous system directly or indirectly. A brief account of various kinds of exotoxins which affect the nervous system in a detailed manner are provided in Table 5.2. Two characteristic bacterial neurotoxins are botulinum (BoNT) and tetanus (TeNT) toxins secreted by *C. botulinum* and *C. tetani* (Popoff and Poulain 2010). The neurotoxins exclusively target the central or peripheral nervous system and induce neurological disorders like botulism and tetanus. The clostridial neurotoxins act on motor neurons, and inhibit acetylcholine secretion at the myo-neural synapses leading to paralysis (Poulain and Doussau 2020).

Botulinum Toxin

Clostridium botulinum produces a variety of botulinum toxins. The BoNT-secreting strains of *C. botulinum* are classified into four groups based upon their genotypic and phenotypic characteristics. Till date, seven BoNT toxins (namely BoNT/A, B, C, D,

Table 5.2 Function/effect of various kinds of toxins on interaction with their respective host receptors

Toxin	Bacteria	Host receptor	Function
Botulinum neurotoxin	*Clostridium* spp.	Synaptotagmin SV2, gangliosides	Inhibits acetylcholine release
Tetanus neurotoxin	*Clostridium tetani*	GPI-anchored protein	Inhibits neurotransmitter release (GABA)
Pneumolysin	*Streptococcus pneumoniae*	Cholesterol	Neuronal apoptosis
Enterotoxin	*Clostridium perfringens*	–	Forms pores in epithelial cells, creating ulcers
Cholera toxin	*Vibrio cholerae*	Ganglioside GM_1	5-HT release causing diarrhea
Epsilon toxin	*Clostridium perfringens*	–	Activates glutamate release
Staphylococcal toxin	*Staphylococcus aureus*	MHC class II	Stimulates 5-HT_3 receptor-causing emesis
Cereulide	*Bacillus cereus*	5HT_3 receptor	Stimulates 5-HT_3 receptor-causing emesis
Toxin A	*Clostridium difficile*	Membrane glycoprotein	Releases inflammatory molecules
Heat-labile/stable enterotoxin	*Escherichia coli*	Ganglioside GM_1/ guanylate cyclase	Diarrhea/stimulation of vagus nerve

E, F, and G) have been identified based upon their sequence and antigenic effects (Padda and Tadi 2021). BoNTs are expressed as a precursor protein, which are converted into active toxins by exogenous bacterial proteases or intestinal digestive proteases. BoNT in its native state is a heterodimer consisting of a light (L) chain (~50 kDa), and a heavy (H) chain (~100 kDa) linked by disulfide bridge. The structure of BoNTs chains can be divided into three domains—(1) L-chain containing α-helices, β-strands, and a catalytic zinc-binding domain, (2) H-chain contains two long twisted α-helices at N-terminal, and (3) two C-terminal subdomains called HCN and HCC for receptor recognition (Padda and Tadi 2021). Further the central domain of L-chain and half N-terminal domain of H-chain is highly conserved in clostridial neurotoxins. The central L-chain domain contains a consensus sequence His-Glu-X-X-His, which is a zinc metalloprotease active site (Poulain and Popoff 2019). The HCC domain is highly divergent and responsible for affinity toward multiple receptors and gangliosides. BoNTs enter via the digestive route and undergo transcytosis via intestinal mucosa to enter the bloodstream. Upon diffusion, BoNTs interact with motor neurons. BoNTs recognize three specific receptors based upon the BoNT type. BoNT/A, C, E, F interact with vesicle protein, SV2 isoforms while BoNT/B and G target synaptotagmin I and II. The BoNTs-receptor complex mediates acidified clathrin-coated vesicular endocytosis of BoNTs. The acidified vesicles stimulate translocation of L-chain into the peripheral nervous system where it blocks the release of acetylcholine resulting in flaccid paralysis. The metalloprotease domain of L-chain targets members of

SNARE proteins. SNARE proteins play a vital role in exocytosis of neurotransmitters. Both BoNTs and TeNT participate in proteolytic cleavage of SNARE proteins to induce blockade of neurotransmitter release at the synapse. BoNT/B, D, F, and G and TeNT interact with synaptobrevin, or VAMP, BoNT/A and E cleave SNAP25, and BoNT/C1 cleaved SNAP25 and syntaxin (Poulain and Doussau 2020).

Tetanus Toxin

Unlike BoNTs, only one type of tetanus toxin is expressed by *C. tetani*. Both BoNTs and TeNTs share a common structure with an amino acid sequence identity ranging from 34% to 97%. *C. tetani* colonizes in the wounded area and releases TeNTs that diffuse directly into the extracellular fluid (Poulain and Doussau 2020). TeNTs target all types of nerve endings, i.e., sensory, motor, and adrenergic neuronal endings, but are transported through motor neurons (Dong et al. 2019). TeNTs bind to the GPI-anchored membrane protein called Thy-1. Unlike BoNTs, TeNTs are internalized by endocytic vesicles which are not acidified. The TeNT vesicles retrogradely transport TeNTs with the help of microtubules to the cell body of neurons in the spinal cord (Dong et al. 2019). The retrograde transfer of TeNTs is mediated by presence of neurotrophin receptors such as p75NTR in vesicular organelles. The C-terminal domain of TeNTs mediates the retrograde transport and reaches the target neurons by transsynaptic migration. The target neurons of TeNTs are inhibitory interneurons involved in regulation of motor neurons. Upon entry into inhibitory neurons, TeNT vesicles are acidified, which permit the delivery of L chain into cytosol, where the L-chain inhibits the release of glycine and GABA (Cai et al. 2021).

Pore-Forming Toxins (PFTs)

Pore-forming toxins or PFTs are bacterial cytotoxins which destroy the host cell membrane by forming pores. PFTs are responsible for toxin-mediated cellular damage, nutrient uptake, evading immune cells, internalization, and dissemination of pathogens. A large group of pathogens such as *Streptococcus* sp., *S. aureus*, *E. coli,* and *M. tuberculosis* utilize PFTs for their pathogenesis (Los et al. 2013). *S. aureus* produces an arsenal of PFTs which work as virulence factors. PFTs are divided into two groups, α-PFTs and β-PFTs based upon the secondary structure of the membrane spanning elements (Hu et al. 2021). PFTs are transmembrane proteins divided into six families (three α-PFTs and three β-PFTs families). The colicin, ClyA, and actinoporin families belong to α-PFTs' superfamily; while hemolysin, aerolysin, and CDC belong to β-PFTs superfamily (Dal Peraro and Van Der Goot 2016). All the six families comprising of the colicin, ClyA, hemolysins, and CDC families have been extensively studied in relation to bacterial infections. The colicin, ClyA, and CDC are lipid and cholesterol-binding toxins while hemolysins target membrane proteins. The interaction with membrane components initiates oligomerization of PFTs, which is preceded by the exposure of the hydrophobic region for integration into the host cell membrane creating porous structures in the host cell membrane. The membrane permeability varies among PFTs, as they act like host membrane channels while increasing the membrane permeability (Brito et al. 2019).

Colicin Family

Colicins are produced by *E. coli* to kill other bacterial species by pore formation in the inner bacterial membrane. They increase the virulence of *E. coli* by invading the niche and decreasing the ecological competition. The structure of colicin contains an inside-out protein fold, i.e., a hydrophobic helical hairpin within amphipathic alpha helices. Upon insertion, colicin oligomers rearrange as an "umbrella" structure to form a nonspecific voltage-gated channel. The voltage-gated channel leads to membrane depolarization and ATP depletion. The umbrella model has been reported in case of colicin A, colicin E1, colicin A23, and colicin Ia. The general targets of colicins are inner membrane lipid bilayer (Chérier et al. 2021).

ClyA Family

The Cytolysin A (ClyA) family of toxins includes ClyA/HlyE (*E. coli*, *S. flexneri*, and *S. enterica*), and *B. cereus* NHE-Hbl complex. The ClyA family of PFTs interacts with cholesterol molecules embedded in the cellular membrane. In the solution state, ClyA is an elongated α-helical protein with a short β-hairpin called the β-tongue (Li et al. 2021). In the presence of cholesterol, the β-tongue is embedded into the lipid bilayer. The tripartite PFT secreted by *B. cereus* contains two components called hemolysin BL (Hbl), and nonhemolytic enterotoxin (NHE). Hbl is composed of three subunits: B, L1, and L2; while NHE contains subunits A, B, and C. All the subunits of Hbl and NHE assemble in a linear manner to form a pore in mammalian membrane (Bräuning and Groll 2018).

Hemolysin Family

Hemolysins play an instrumental role in pathogenesis of a variety of Gram-positive bacteria such as *S. aureus* and *C. perfringens*. Hemolysins promote evasion of the immune system, bacterial dissemination (in cases of biofilms and internalized pathogens). The prevalent targets of hemolysins are immune cell surface proteins such as chemokine receptors and complement receptor. Hemolysins were first identified in *S. aureus* which causes pneumonia and sepsis (Bubeck Wardenburg et al. 2007). *S. aureus* hemolysins are composed of either single component like α-hemolysin (Hla) or two component such as γ-hemolysins (HlgAB and HlgCB) and leukocidins (Panton-Valentine leukocidin (PVL), LukED, and LukAB). *S. aureus* hemolysins, Hla and PVL are prime virulence factors in case of lung infections caused by community-associated methicillin-resistant *S. aureus* (CA-MRSA). Various clinical strains of *S. aureus* such as *S. aureus* Newman have been investigated for the clinical relevance of Hla and PVL in staphylococcal lung infections (Wardenburg et al. 2007). The *agr* QS system predominantly regulates the abovementioned PFTs together with proteases (staphopain, aureolysin) and Spa protein (Le and Otto 2015). The staphylococcal PFTs share similar structural fold and assembly with clostridial PFTs such as necrotic enteritis toxin B, VCC55, and δ-toxins. Single-component PFTs assemble to form heptameric pores while bi-component PFTs form octameric pores (Wardenburg et al. 2007).

Enterotoxins

Enterotoxins target intestinal epithelial lining causing ulceration, diarrhea, and food poisoning. Enterotoxins can be cytotoxic (shiga and shiga-like enterotoxin),

cytotonic (heat-labile enterotoxins), or both (staphylococcal enterotoxins A and B) in nature. Enterotoxin such as cholera toxin stimulates hypersecretion of water and electrolytes resulting in diarrhea. The shiga toxins facilitate *Shigella* spp. to penetrate the mucosal surface of the colon-causing ulcers. While pyrogenic enterotoxins such as staphylococcal enterotoxins A and B (SEA and SEB) cause staphylococcal/ streptococcal food poisoning, and have cytotoxic effects on immune cells (Aktories and Barbieri 2005; Bharati and Ganguly 2011). A brief description of the host–pathogen interaction involving medically relevant enterotoxins is mentioned as follows.

Heat-Labile Enterotoxin

Heat-labile enterotoxins include Cholera toxin (CTX) and *E. coli* enterotoxins (Elt). CTX and Elt are well known as diarrheal toxins that reduce absorption of water by the intestinal epithelial lining in an irreversible manner (Dubreuil 2012). Both CTX and Elt are large multimeric A-B type toxins. CTX and Elt are hexamers composed of an enzymatic A subunit together with five receptor-binding B subunits (AB_5). The B subunits form a five-membered ring, which is attached to the enzymatic (A) subunit by the extended helical A2 domain of A subunit. On the other hand, the A1 domain of A subunit is a globular enzyme which catalyzes ADP-ribosylation of G proteins. The A subunit activates G proteins and adenylate cyclase signaling cascade. While the B subunits bind specifically to gangliosides such as GM1, Lewis X, and Lewis Y (Bharati and Ganguly 2011; Dubreuil 2017). The mechanism of action followed by CTX and Elt is described in Fig. 5.6. Moreover, CTX has been identified to induce an iron-deficient metabolic niche by sequestering heme rings and fatty acids to promote *V. cholerae* proliferation (Rivera-Chávez and Mekalanos 2019).

Heat-Stable *E. coli* Enterotoxins

E. coli secretes another type of diarrheal enterotoxins that retain their 3D structure at temperatures as high as 100 °C. The heat stable enterotoxins or STs are small cysteine-rich secretory peptides produced by enterotoxigenic *E. coli* (ETEC). Unlike Elts, STs interact with guanylate cyclase C and sulfatides in a reversible manner. Three STs namely STa (STaP and STaH), EAST1, and STb have been experimentally identified as ETECs. The STa and EAST1 share a similar mechanism of action while STb triggers serotonin production together with activation of chloride ion channels, and blocking of sodium ion channels (Dubreuil 2017).

Shiga and Shiga-Like Enterotoxins

Shiga toxin (Stx) is a highly potent enterotoxin and is associated with hemolytic uremic syndrome. It is produced by dysentery-causing *Shigella dysenteriae* and severe enterohemorrhagic infection-causing shiga toxin-producing *E. coli* (STEC). Stx can be divided into two groups Stx1 and Stx2. Stx1 is found in *S. dysenteriae* and some *E. coli*, while Stx2 is expressed specifically by *E. coli*. Stx1 and Stx2 share a similar structure and mode of action, although they are antigenically distinct (Melton-Celsa 2014). Like CTX, Stxs are AB_5 type toxins where monomeric A subunit binds to ribosomes and pentameric B subunit interacts with globotriaosylceramide (Gb3) of the epithelial cells. Upon interaction of B subunit with Gb3, enzymatic domain of A subunit is trafficked in a retrograde manner within

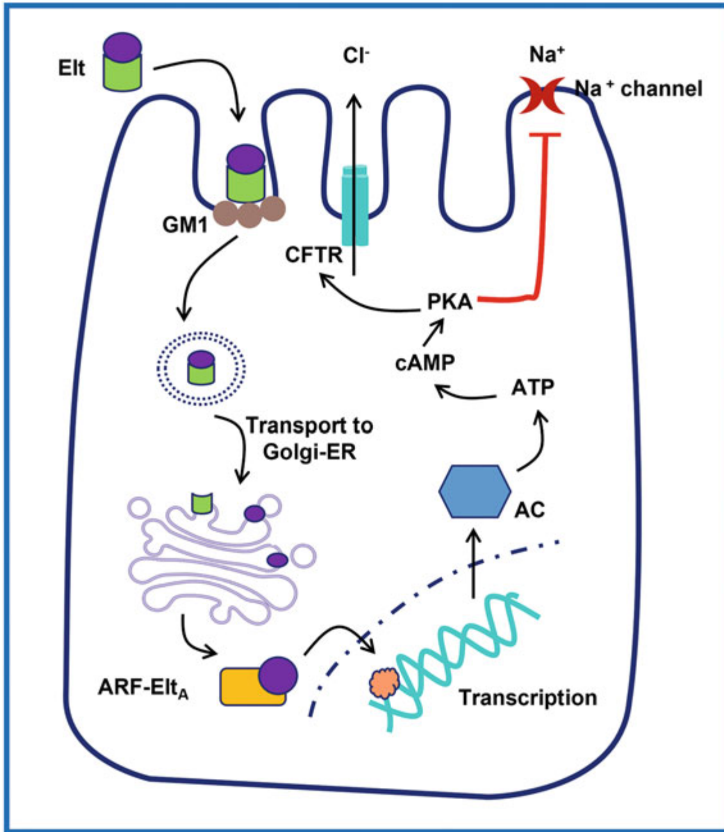

Fig. 5.6 Schematic representation of Let interaction with ganglioside present on the epithelial cells, and blocking of sodium channels via PKA signaling

the cell until it reaches to the endoplasmic reticulum (ER). Upon reaching the ER, the enzymatic domain is released and destroys ribosomes resulting in blockade of protein synthesis. Moreover, Stxs induce chemokine response leading to severe inflammatory stress. Stxs produced by STECs lead to dire manifestations such as hemolytic uremic syndrome where blood clots damage kidney blood vessels resulting in kidney failures (Lee and Tesh 2019).

Bacillus Enterotoxins

Bacillus produces two medically relevant enterotoxins, cereulide and the nonhemolytic enterotoxin (NHE), part of tripartite α-PFTs. The latter has been explained in the above section. Cereulide is an emetic toxin that leads to emesis syndrome in humans. Structurally it is a cyclic dodecadepsipeptide like the potassium ionophore valinomycin (Singh and Lad 2021). The 1.17 kDa cyclic peptide (d-*O*-Leu-d-Ala-l-*O*-Val-l-Val)$_3$ is highly hydrophobic with a p*I* value of 5.52 (Ramarao et al. 2015). Cereulide is synthesized by a nonribosomal peptide synthetase gene cluster called

ces during the stationary phase of the bacterial growth cycle (Tuipulotu et al. 2021; Ehling-Schulz et al. 2019). The toxin is predominantly found in dairy and starchy products such as milk and rice (Singh and Lad 2021). Owing to its hydrophobicity, cereulide is easily absorbed in the intestine and accumulates in the liver, kidney, or muscle tissues (Jovanovic et al. 2021). The leading cause of emesis by cereulide is based upon interaction of cereulide with serotonin 5-HT3 receptors (Tuipulotu et al. 2021). The serotonin 5-HT3 receptors are expressed in the stomach and small intestine to mediate gut-to-brain signaling through the vagus nerve. Upon activation of the vagus nerve by cereulide-serotonin 5-HT3 complex, emesis (vomiting) is triggered. A study on house musk shrew infected with emetic toxin showed that antagonists of 5-HT3 receptors or severing of vagus nerve blocks cereulide-induced emesis (Tuipulotu et al. 2021).

Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SE) are responsible for food poisoning and toxic shock syndrome. The SEs produced by *S. aureus* can be divided into two groups: (1) classical SEA-SEE group and (2) newer SEG-SE/Y group (Pinchuk et al. 2010). SEs are also known as superantigens (PTSAgs) due to their ability to bind with class II MHC molecules present APCs leading to activation of T cells. T cell activation results in a cytokine storm eliciting an acute toxic shock. The SEs cause an emetic response by inducing production of serotonin or 5-hydroxy tryptamine (5-HT), resulting in stimulation of the vagus nerve as explained above (Fisher et al. 2018).

5.3.1.3 Other Proteins Involved in Host–Pathogen Interactions

Apart from the bacterial proteases and toxins, several proteins that inherently bind to biomolecules (other than proteins) have been documented and are known interactors of immunity-associated proteins (Johnson 2018). Such proteins are often secreted by the bacterial system into the host tissue environment with the aim of targeting the host defense system. One such protein is histone-like DNA-binding protein (Hup) of *Helicobacter pylori* (Jaiswal et al. 2018). Hup protein is a DNA-binding protein, which is actively involved in nucleic acid-dependent cellular activities such as replication, recombination, and DNA repair mechanisms. The protein is also known to protect bacterial genome from oxidative stress and is explicitly secreted by T4SS into the gastric epithelia during *H. pylori*-associated inflammatory infections (Agarwal et al. 2021, 2022). Hup protein has been reported as a potent interactor of host protein involved in sumoylation to modulate host immune system. SUMOylating proteins, belong to SUMO family of proteins, which mainly interact with nuclear proteins involved in DNA repair and chromatin arrangement. SUMO-1 has been recently reported as an active partner of *H. pylori* Hup protein with the help of structural studies (Jaiswal et al. 2019). The interaction of Hup and SUMO-1 suggests the importance of Hup in *H. pylori* infections and as a potential drug target (Jaiswal et al. 2019; Raj et al. 2020, 2021). Similarly, secretory lipoprotein such as SaeP from *S. aureus* has been studied for the affinity toward immune-associated proteins via interaction with extracellular DNA (eDNA) present in the bacterial biofilms. Such interactions have been shown to promote bacterial biofilm formation and as potential targets for novel antibiofilm therapies (Kavanaugh et al. 2019).

Lipoproteins present in the outer membrane vesicles (OMV) of other Gram-negative bacteria such as *Vibrio* and *Neisseria* also mediate infection by targeting host guanylate-binding proteins and activation of inflammasome (Santos et al. 2018).

5.4 PPIs in Viral Infections

Viruses are biological nanoparticles where nucleic acid (DNA/RNA) is encapsulated in a proteinaceous cage-like structure known as capsid. Viruses require living cells for their propagation and cause a majority of diseases in humans. Based upon the type of nucleic acid and encapsulation, viruses are classified into 17 genera. Over 200 viruses are known to infect humans. Viruses together with bacteria have been a major cause of pandemics over the years. Viral infections can be deadly such as HIV-AIDS, MERS, Ebola with associated morbidity and high-mortality rates. The severity of viral infections depends on the evolution of viruses which results either in less virulent strains (common cold virus and influenza virus) or highly virulent strains (SARS-COV2 and MERS strains) (Breitbart and Rohwer 2005). In general, the viruses, like influenza virus, possess an arsenal of nonstructural proteins (NS proteins) that show very high structural diversity and internal disorders (Jaiswal et al. 2020). The pathogenesis of viruses depends upon how the host interacts with viral proteins and nucleic acids. Moreover, the constant changing, and evolving strains of viruses lead to evolution and adaptation in host proteins involved in host–pathogen interaction in viral infections. As covering all the viral proteins and their interactions is out of the scope of this book, this section will discuss a family of the clinically relevant viral protein–host interactions.

5.4.1 Viral Capsid Proteins and Their Molecular Partners

Capsid is the outer envelope of the cellular structure of a virus. It is mainly made up of protein; however, in some viruses the capsid is surrounded by an additional lipid bilayer. Capsid protein promotes viral attachment to the host cells and their entry inside the cells (Wißing et al. 2021). The entire process is an amalgamation of several steps taking place simultaneously. These steps include: cell recognition, entry, and uncoating for internalizing nucleic acid. Animal viruses such as poliovirus and human rhinovirus (HRV) are nonenveloped, and their attachment and uncoating are mediated by receptor binding or acidic pH. Various HRV2 uncoating intermediates where acidic pH-induced transition of HRV2 virions into an elongated porous intermediate called A-particle have been elucidated by X-ray and cryo-electron micrography (Pickl-Herk et al. 2013). In contrast, enveloped animal viruses utilize a two-step process for delivering nucleic acid into the host cell. Primarily they interact with cell-surface receptors to initiate the fusion of viral and host membranes (Más and Melero 2013). Wang et al. have successfully characterized the differences between spike glycoprotein present on MERS-CoV and dipeptidyl peptidase 4 (DDP4) (Wang et al. 2016). Similarly, host–cell interactions with various other

viral proteins such as Spike2 protein (SARS-Cov-2), E2 protein (Papillomavirus), NS2 protein (Hepatitis C virus), and HA-1 (Influenza virus) have been characterized in recent years (Mayer et al. 2019). These proteins are gravely important for initiation of viral infections and undergo constant mutation for escalating the viral infectivity. In addition to protein–protein interactions, viral/host glycans are of paramount importance in viral attachment.

5.4.1.1 Spike Protein and Its Molecular Partners

Spike proteins are type I transmembrane proteins expressed in the viral capsid of coronaviruses (Belouzard et al. 2012). These proteins are rich in Ser/Thr residues and are involved in N-glycosylation. They assemble in a trimeric conformation imparting a crown-like appearance (corona) (Stertz et al. 2007). The ectodomain structure can be divided into two regions: an amino-terminal region called S1, and a carboxy-terminal region called S2. The S1 and S2 domains are involved in receptor binding and host cell fusion. S1 region is highly variable and diverges forming novel strains of a coronavirus, while the S2 subunit is highly conserved. The characteristic structural feature of spike protein is the alpha-helical coiled-coil structure present in the carboxy terminal region of the protein similar to the influenza hemagglutinin protein (HA protein) which is the key characteristic feature of class I fusion protein family. Moreover, spike protein also contains two heptad repeats in their S2 domain (Li 2016). A heptad repeat consists of repetitive seven-residue peptides, where first and fourth residues are hydrophobic and participate in the fusion process by forming the coiled-coil structure. As mentioned earlier, viral capsid proteins exhibit affinity toward glycans as well. Hence, the molecular partners of spike proteins range from host proteins to glycan moieties present on the cell surface. The S1 domain actively participates in both glycan and protein binding (Watanabe et al. 2020).

The molecular partners of spike proteins are present within each genus. For example, spike proteins of MERS coronavirus (MERS-CoV) and MHV have high affinity toward DDP4 and CEACAM1 respectively; while HCoV-NL63, SARS-CoV-1, and SARS-CoV-2 interact with the angiotensin-converting enzyme 2. The trimeric spike protein interacts with the DDP4/CEACAM1/ACE2 receptors and induces membrane fusion for transfer of genetic material into the host cells. Owing to their relevance in infection, viral spike and abovementioned host receptors are often used as immunogens for designing vaccines and generating neutralizing antibodies. Glycosylation plays an important role in interaction and immune evasive action of spike proteins in coronavirus infections (Zhao et al. 2020). All spike proteins possess canonical sequon—NXS/T (where $X \neq P$) for N-glycosylation (Watanabe et al. 2020). The viral entry in case of enveloped viruses proceeds by formation of virions which are basically endosomal vesicles carrying genetic material. The virions of CoV bud into the ER lumen and traverse the secretory pathway (Stertz et al. 2007). Moreover, it has been proposed that CoVs are capable of acquiring a host glycan coat which facilitates immune evasion (Zhao et al. 2020).

Ace2–Spike (SARS) Interactions

ACE2 or angiotensin-converting enzyme 2 is an integral membrane protein. It is a metalloproteinase involved in regulation of the renin-angiotensin system and tends to show uncompetitive binding affinity toward SARS-CoV-1 and SARS-CoV-2 spike proteins (Tikellis and Thomas 2012). Similar to spike N-glycosylation, polymorphic ACE2 genes have been identified in the human population that reshape the ACE2–Spike interaction via varied glycosylating patterns (Hoffmann et al. 2020). The S1 carboxy-terminal domain (S1 CTD) is also involved in interaction with ACE2 protein. The structure of SARS-CoV S1 CTD complexed with ACE2 has been elucidated. The S1 CTD contains a receptor-binding domain (RBD) made up of five-stranded antiparallel β sheets (Ou et al. 2017). The RBD forms a concave surface for proper binding with ACE2. The RBD identifies the membrane-distal peptidase domain of ACE2 which contains several virus-binding motifs (VBFs) (Zhao et al. 2020). VBFs are present away from the catalytic site, hence not interfering with the enzymatic activity of ACE2. The hotspot residues of ACE2, Lys31 and Lys353, which consist of a salt bridge in a hydrophobic vicinity are critical for the virus-receptor binding. Further, residues 479 and 487 in SARS-CoV S1 CTD are known to be under high selective pressure and involved in cross-species transmission of virus (Cai et al. 2020). This was identified during the initial SARS-CoV epidemic where civet-to-human transmissions and mutations in key-evolving residues enhanced the binding of SARS-CoV to human ACE2. Such mutations led to strengthening and enhanced binding affinity of S1 CTD-ACE2 complex during epidemic. Similar conditions have been identified in the recent outbreak of SARS-CoV-2, where mutations in the spike protein gave rise to novel strains like alpha, beta, gamma, delta, and omicron strains due to bat-to-human and human-to-human transmissions (Li et al. 2005; Hossain et al. 2021; Dawood 2020).

ACE2–Sipke (HCoV-NL63) Interactions

HCoV-NL63 is an alphacoronavirus unlike SARS-CoV, which belongs to the betacoronavirus family. Both recognize ACE2, although they possess structurally divergent RBD domains. The S1-CTD domain of HCoV-NL63 is a β-sandwich consisting of two three-stranded antiparallel β-sheets. The loops are discontinuous in contrast to SARS, where RBD is a long continuous subdomain, where Lys353 is a critical hotspot for both viruses (Li 2016).

DPP4–Spike (MERS) Interactions

Similar to SARS S1 CTD, MERS-CoV S1 CTD is divided into two subdomains, RBD and a core structure. The difference in protein receptors of both the viruses is due to the variation in RBD domain. The MERS-CoV RBD is a four-stranded antiparallel β-sheet, which forms a flat structure for DPP4 binding. DPP4 binds with spike protein in a homodimer form. The monomeric form for DDP4 consists of a hydrolase domain and a β-propeller domain. The VBMs in DDP4 are located on the exterior surface of the β-propeller domain and pose a barrier for cross-species transmissions of MERS-CoV. In case of MERS-CoV epidemic, a camel-to-human

and bat-to-human transmission have been suggested. In the case of bats, a surface protein, HKU4 has been recognized as a molecular partner of DPP4 (Li 2016).

5.5 PPIs in Fungal Infections

Fungi causes life-threatening infections in humans and accounts for approximately 1.7 million deaths annually. Fungal infections have been associated with high morbidity and mortality rates. In general, cases of fungal infections are recorded in aged people and immunocompromised patients. Use of immune-modulating drugs, biofilm formation on medical devices, and dietary choices are major reasons behind increasing fungal infections. Moreover, identification of novel antifungal-resistant strains is limited, as antifungal drugs have elevated the risk associated with fungal infections. As of today, only three distinct classes of drugs—azoles, echinocandins, and polyenes are qualified as active antifungal drugs. Most of the fungal infections in humans are caused by variants of *Aspergillus*, *Candida*, and *Cryptococcus* (Köhler et al. 2015). Moreover, fungal infections are often coassociated with bacterial or viral infections. For instance, a rare fungal infection, mucormycosis (caused by *Rhizopus* or *Mucor* species), together with invasive candidiasis, and aspergillosis have been associated with COVID-19 patients. Further, *Candida* has been ranked as a serious threat together with MRSA and vancomycin-resistant *Enterococci* by the US Center for Disease Control and Prevention. *Candida* species such as *C. auris*, *C. glabrata* have been discovered to be resistant to one or all the classes of antifungal drugs. Four vital criteria should be fulfilled by fungus to infect a human. These are as follows: (1) ability to grow at elevated temperature, (2) ability to penetrate host tissue barriers, (3) ability to decompose, mimic, and absorb components of host tissue, and (4) ability to evade/attack host immune system. Like other pathogenic infections, the pathogenesis of fungal infections is regulated by the host–pathogen interactions. This section explains various PPIs involved in host–fungus interaction with the help of *Candida albicans* infections (Kainz et al. 2020). *Candida albicans* is a polymorphic fungus, i.e., it can grow as yeast cells, pseudohyphal form, and true hyphal form. The intensity of *Candida* infection depends upon the morphological diversity and a series of virulence factors described as follows:

5.5.1 HPIs Involved in *Candida* Infection

The epithelial tissue is the first line of defense against *C. albicans*. Hence, invasion of host epithelial cells is the first and foremost action during the fungal infection. *Candida* invasion through epithelial cells has been explained by two molecular mechanisms—passive fungal-induced endocytosis, and active penetration. Both processes are mediated by special structures such as invasins (agglutinin-like sequence 3(Als3), Ssa1) or hypha. The molecular mechanism depends on the morphology, invasion stages, and lineage of epithelial cells. The active penetration is the predominant method of invasion, while endocytosis takes place only in

preliminary stages, i.e., within 4 h of invasion. Quorum sensing (QS)-mediated hypha formation also modulates the invasion with the help of cAMP/PKA and MAPK signaling cascades. A hypha-deficient strains with dead mutants of fungal proteins involved in cAMP/PKA signaling cascades have shown reduced ability of fungal invasion. Other than invasions, *Candida* expresses several proteases, lipases, phospholipases, and a toxin called candidalysin which are involved in invasive candidiasis (Pappas et al. 2018).

5.5.1.1 HPIs Involving Fungal Invasins: Als3 and Ssa1

Invasins are fungal proteins that promote penetration into host cells during pilot stages of infection. Till date, two *Candida* proteins, Als3 and Ssa1 have been identified as invasins. Invasins are present on the hyphal surface and bind to E-cadherin (epithelial cells), N-cadherin (endothelial cells), and EGF receptors to induce clathrin-mediated endocytosis. The invasins–cadherin interaction stimulates rearrangement of actin and recruitment of clathrin, dynamin, and cortactin at the site of entry. The internalization of *C. albicans* takes place in a zipper-like process, where the host cell produces pseudopods which encircles the fungus and engulfs it into the host cell (Ciurea et al. 2020).

Als3 is a hyphal-associated protein belonging to the *Als* gene family. It is a glycophosphatidylinositol (GPI)-anchored cell surface protein. The structure of Als3 protein is divided into three domains: (1) N-terminal domain composed of a signal peptide, immunoglobulin-like domain, and a Thr-rich antiparallel β-sheet domain, (2) central domain composed of a Ser/Thr-rich variable 36-amino acid tandem repeat; and (3) heavily glycosylated C-terminal domain which is also rich in Ser and Thr. The N-terminal and central domain of Als3 play a role in adherence, while the C-terminal domain is anchored to GPI-associated cell surface proteins (Liu and Filler 2011). It is predicted that due to the anchorage sequence present in the C-terminal domain, it is cleaved when Als3 is exported into the host cell. As the N-terminal domains of cadherin and Als3 are similar in structure, the interaction between Als3 and cadherin is believed to follow the same mechanism as that of cadherin–cadherin (Ciurea et al. 2020). Besides fungal-induced endocytosis, Als3 is also involved in perpetuating epithelial anchoring mediating active penetration mechanisms. The active penetration via Als3 does not involve interaction with E-cadherins. Wachtler et al., showed impaired invasion of E-cadherin-deficient HeLa cells by Als3Δ mutant, and also observed reduced invasion of Als3Δ mutant compared to wild type in cases of cytochalasin D-treated TR-146 cells (Wächtler et al. 2012).

Ssa1 is also expressed on the hyphal surface and binds to E- and N-cadherins like Als3. It is a heat shock protein belonging to the HSP70 family. Along with their function as molecular chaperones, members of the HSP70 family are also involved in translocation across membranes (Ciurea et al. 2020). Ssa1 contains two domains: N-terminal ATPase domain and C-terminal peptide-binding domain. The N-terminal domain is involved in cellular processes via ATP hydrolysis (Mayer et al. 2013). The C-terminal domain is further divided into two subdomains: a hydrophobic β-sandwich cavity for peptide binding, and a helical lid-like subdomain that

mediates ATP-dependent substrate peptide processing. Attenuation of endocytosis has been observed from several studies based on Als3 and Ssa1 deletion mutants (Sun et al. 2010). Both Als3 and Ssa1 are believed to stimulate the same pathway for endocytosis. Sun et al., studied the relationship between Ssa1 and Als3 by constructing double (Ssa1Δ/Δ-Als3Δ/Δ) and single mutants (Ssa1Δ/Δ and Als3Δ/Δ), and observed that the host–cell interaction reduced at an earlier time point in double mutants as compared to single mutants (Sun et al. 2010).

5.5.1.2 HPIs Involving Fungal Enzymes

Candida produces a variety of hydrolytic enzymes such as secreted aspartate proteases (Saps and yapsins), phospholipases (PL), and lipases involved in fungal invasion, tissue damage, and activation of innate immune response. These enzymes promote the active penetration process of fungal invasion together with the mechanical force exerted due to elongation of the hypha (Perlin et al. 2017). Hence, metabolic and molecular investigations are promising approaches to reinforce pharmacological targeting (Gupta et al. 2022).

Candidal aspartate proteases are involved in a wide array of host–pathogen interaction during fungal infections. Two types of aspartate proteases—Saps/Candiapepsin and Yapsins have been identified in *Candida* sp. (Monika and Zbigniew 2017). Saps or candidapepsin are a family of aspartate proteases expressed in *C. albicans*. While Yapsins are specifically expressed in *C. glabrata*, where 11 Yapsins (Yps)-encoding genes have been identified. Saps have been extensively characterized over the years compared to yapsins. The Saps protease family contains ten types of proteases divided into two subfamilies—Saps 1–3 and Saps 4–6 (Wu et al. 2013). Saps are involved in invasion, evasion, and phagocytosis of host cells. They are positively involved in the active penetration of *C. albicans*. Studies have shown that inhibition of Saps using aspartic protease inhibitor (pepstatin A) reduces the invasive capacity of *C. albicans* strains (Monika and Zbigniew 2017). Saps mediate the invasion process by three different mechanisms: (1) degradation of E-cadherins present in cell-cell adherens junction, (2) degradation of mucins present in tissue barrier, and (3) promotion of hypha formation. Saps 1–6 are responsible for degradation of E-cadherins, and other host proteins; while Saps 4–6 promote formation of hypha. In addition to the invasive activity of Saps, Saps have been characterized to activate interleukin-1β precursor thereby stimulating an inflammatory response (Schaller et al. 2005). Saps 4–6 have been investigated for their role in induction of keratinocyte response, reduced E-cadherin, and disbandment of cell-cell adhesions. Saps are also involved in internalization of *Candida albicans* by degradation of lysosomes leading to the activation of caspase-mediated apoptosis and tissue damage.

Beside fungal invasion, proteases are key regulators of mechanisms involved in growth, proliferation, and immune evasion during candidiasis. The aspartate proteases are involved in fungal cell wall maintenance and remodeling during transition between *candida* polymorphs (Schaller et al. 2005). Sap9, Sap10, and yapsins have been reported to degrade chitin synthases, yeast-form cell wall protein-1 (Ywp1), Als2 proteins, which are involved in cell wall formation. While Yps1 and

Yps7 are involved in cell–cell interactions. Moreover, Yps are also involved in survival under stress conditions as shown by inability of Yps mutant to survive the antifungal macrophage response in animal models of systemic candidiasis. The proteases also elevate fungal survival by promoting pathogenic biofilms. In comparison to planktonic fungal cells, a remarkable upregulation of Saps-encoding genes has been observed in Candida biofilms (Yu et al. 2016). The two most important Saps involved in biofilm formation are Sap5 and Sap6. Sap8 also maintains Candida biofilms by degrading signaling mucin Msb2, which is involved in Cek1 MAPK pathway. Immune evasion is another pathological process which is modulated by aspartate proteases by *Candida* yeast cells (Yang et al. 2014). The proteases show affinity toward immunoglobulins, specifically IgA, and complements. The protease hydrolyzes Igs and complements impairing their action against yeast cells. Sap1–3 have been experimentally proven to degrade complement proteins C3b, C4b, and C5 and prevent phagocytosis. Saps are also involved in impairment of pattern recognition by host immune cells. Candida proteases also exhibit a stimulatory effect on blood coagulation, and the kinin formation process. Saps can activate serine proteases zymogen, a class of blood coagulation factors. Through in vitro experiments, it has been proven that *C. albicans* Saps can activate factor XII, factor X, and prothrombin, which are essential for blood coagulation (Rapala-Kozik et al. 2018). The activation of factor XII initiates their adsorption through blood vessels together with a zymogen, prekallikrein (PK), and high-molecular mass kininogen (HK) (Yang et al. 2014; Rapala-Kozik et al. 2018). PK in its active form (kallikrein) digests HK and produces bradykinin, which belongs to the family of vasoactive and inflammatory peptides called kinins. Increase in concentration of kinins in the vascular system promotes permeability and migration of immune cells to the site of infection. In addition to leukocyte recruitment, kinins increase the availability of nutrients and dissemination of pathogens. Two mechanisms have been widely accepted for Saps-mediated kinin production: (1) activation of factor XII as mentioned above, and (2) proteolytic cleavage of kininogens. Saps are also involved in hydrolytic cleavage of proteases inhibitors (α2-macroglobulin and cystatin A), and antimicrobial peptides such as histatin-5, cathelicidin—LL-37 (Rapala-Kozik et al. 2018).

Other than, Saps hydrolytic enzymes such as phospholipases (PL) and lipases are key mediators of host–pathogen interaction in *Candida* infections. Phospholipases hydrolyze phospholipids into fatty acids, while lipases hydrolyze lipids (Mustranta et al. 1995). Both PL and lipases are associated with fungal invasion and immune evasion activity (Yang et al. 2014). However, the role of PL and lipases is controversial in case of invasion. Four classes of PL and ten lipases are known to be expressed in *Candida* sp.—PLA, PLB, PLC, PLD, and LIP1–10. Out of these PLs and lipases, PLBs and LIP8 have been experimentally proven to be involved in host–pathogen interactions and PLB1 is abundantly expressed in yeast and pseudohyphal forms (Rapala-Kozik et al. 2018). Attenuated virulence in gene knockout mutants of PLB1Δ/Δ, PLB5Δ/Δ, and LIP8Δ/Δ has been experimentally proven in animal models of candidiasis. Further, it has been reported that the invasive efficiency of PLB1 mutant strains in oral and intestinal epithelial cells are independent of

phospholipase activity. Like PLs, no effect of invasion in presence of a lipase inhibitor ibogaine, thus suggesting for no contribution of lipases in the active penetration mechanisms. The mechanism of PLs-induced invasion can be described by two mechanisms. PLs induce active penetration either by hydrolysis of membrane phospholipids or by facilitating hypha formation from blastospores produced during tissue invasion (Dalle et al. 2010).

5.5.2 Fungal Toxins

The above sections described various membrane proteins and enzymes involved in the pathogenicity of fungal infections in humans with respect to *Candida* infections. Other than the said proteins, fungi produce a wide array of toxins called mycotoxins. Mycotoxins, in general, are inhaled or ingested with dietary products and have a deleterious effect on the human body. Mycotoxins are produced in response to environmental conditions for better survival of fungi. Mycotoxins are either polyketides or peptides in nature (Brown et al. 2021). Till date, two fungal peptide toxins, candidalysin and mucoricin have been identified. Candidalysin is secreted by *C. albicans*, while mucoricin is produced by *Rhizopus delemar*. Candidalysin and mucoricin are encoded by *ECE1* and *RLT1* genes, respectively while polyketides are synthesized by polyketide synthase (Papon et al. 2021). The fungal peptide toxins have similar activity like bacterial cytotoxins, and are also associated with the pathogenicity mediators.

Candidalysin (CL) is an active form of protein/peptide generated from the parent protein—Ece1p by kexin-mediated enzymatic cleavage. CL is secreted by the hyphal form of *C. albicans*, and is amphipathic in nature (Naglik et al. 2019). It adopts an α-helical structure and induces pore formation in the host cell membrane, thereby damaging host tissue (Moyes et al. 2016). CLs also induce immune response by p38, and ERK1/2 pathway-activated cytokine release. G-CSF, GM-CSF, and IL-6 have been identified as predominantly released cytokines in presence of CLs. These cytokines initiate downstream innate immune response and neutrophil recruitment as observed in case of mucosal candidiasis in both murine and human models (Richardson et al. 2018). Moreover, CLs mediate activation of NLRP3 inflammasome, which leads to fungal dissemination by caspase-1-mediated maturation, release of IL-1β, and apoptosis of macrophages/dendritic cells. Further, recently it was shown that CL induces expression of CXCL1 in CARD+ (C-type lectin receptor) microglial cells for neutrophil recruitment in cases of brain-related *Candida* infections (Papon et al. 2021).

Mucoricin is a type of peptide toxin secreted by *Rhizopus delemar* which causes mucormycosis in humans. Mucormycosis is a lethal infection and is often reported in immunocompromised individuals. In recent years, it has been widely associated with COVID-19, leading to severe complications and death of COVID-19 patients. Mucoricin is a 17 kDa ricin (derived from *Ricinus communis*)-like secretory peptide toxin which plays a preeminent role in pathogenesis of *R. delemar*. Mucoricin has a necrotizing effect on several organs. Structurally it contains a lectin-binding domain,

rRNA glycosidase or ribosome-inactivating protein-binding domain. The role of mucoricin has been recently studied by RNAi knockdown expression, structural and computational studies. The studies conducted has revealed that mucoricin mediates inhibition of protein synthesis with the help of N-glycosylase activity and triggers necrosis/apoptosis of nearby cells (Soliman et al. 2021).

5.6 PPIs in Protozoal Infections

Protozoa are single-celled microscopic organisms, either free living or parasitic in nature. Protozoal infections are quite common in poultry animals as well as humans. They are transmitted in humans either through digestive route or via an arthropod vector such as mosquito or sand fly (Esch and Petersen 2013). Some of the common protozoal infections in humans are malaria (*Plasmodium*), African sleeping sickness (*Trypanosoma brucei*), Amoebiasis (*Entamoeba histolytica*), Ascariasis (*Ascaris*), Taeniasis (*Taenia solium*), and Leishmaniasis or Kala azar (*Leishmania donovani*). This section details the host–pathogen interactions involved in protozoal infections using case studies of an important protozoal infection like malaria (Martínez-Girón 2013).

5.6.1 HPIs Involved in Malaria Pathogenesis

Malaria is one of the well-documented protozoal diseases caused by *Plasmodium* sp. It is associated with flu-like symptoms, fever, chills, and fatigue. According to CDC, an estimate of 241 million cases of malaria were recorded worldwide in 2020 with a high mortality rate. *Plasmodium* is transmitted directly into blood via mosquitoes. Among the five malaria-causing *Plasmodium* sp. (*P. falciparum, P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*), *P. falciparum* is the most virulent species. *P. falciparum* manifests three different clinical conditions—asymptomatic, mild, and severe malaria (Milner 2018). Asymptomatic cases are associated with absence of malarial symptoms, while mild and severe cases are accompanied by parasitemia and organ-specific damage, respectively. In severe cases, the parasite can affect the functioning of organs such as the liver, kidney, and brain leading to degenerative implications. The life cycle of malarial parasites involves the hosts and the carrier, *Anopheles* mosquito and human beings. *Plasmodium* is transmitted by *Anopheles* mosquito via skin in its sporozoite form (Frischknecht and Matuschewski 2017). The sporozoite forms primarily invade hepatocytes, where it matures into merozoites and infects mature erythrocytes or red blood cells (RBCs). The malarial parasite feeds on RBCs and grows in a cyclic fashion within the erythrocytes. Inside the erythrocytes, the merozoite wraps itself into a vacuole and matures into a trophozoite and replicates as a schizont (Frischknecht and Matuschewski 2017). The mechanical pressure exerted by formation of schizont leads to RBC rupture and the proliferation cycle continues (Cowman et al. 2012; Acharya et al. 2017). Together with the release of merozoites, several parasitic components are also

released into the plasma, which elicit the host inflammatory responses leading to pyretic condition. The manifestation of disease is a result of a combination of direct and indirect interactions between the host and parasite (Paul et al. 2015). The direct interactions are those where a physical interaction takes place between host and parasitic factor that modulates infection; whereas, indirect interactions are those where either host or parasitic factor is involved in modulation of virulence and related immune response (Fairhurst and Wellems 2006). The host–parasite interactions of the human skin, liver, and erythrocytes are vital for establishment of the infection. Later sections talk about the direct interaction involved at each stage of malarial infection and associated HPIs.

5.6.1.1 *Plasmodium* Interaction with Skin and Liver

Plasmodium is transmitted into the human dermis in sporozoite form. A single mosquito bite results in transfer of 20–200 sporozoites. Within 3h of transmission, these sporozoites enter the circulatory system and traverse through the dermal cells. Several mosquito and human immune response factors catalyze the invasion process (Loubens et al. 2021). For example, mosquito saliva, antihistamines, anticoagulants, vasodilators, and immunomodulators facilitate the survival of sporozoites in dermis. Upon entry into the vascular system, the sporozoites travel to the liver via specialized perforated blood vessels and invade Kupffer cells and hepatocytes through interaction between parasitic proteins such as SPECT1, Perforin-like protein (PLP), and CelTOS and glycosylated scavenger receptor CD68, and CD81 present on Kupffer cells and hepatocytes respectively (Cowman et al. 2012; Acharya et al. 2017). PLPs are an important class of invasive protein in *Plasmodium* that share structural similarity with bacterial PFTs (Peraro and Van Der Goot 2016). Out of five PLPs, PLP2 is known to be functional in the liver as well as blood stage, i.e., merozoites.

HSPGs (heparan sulfate proteoglycans) have also been identified to stimulate the switch from migratory to invasion mode in *Plasmodium* (Dundas et al. 2019) (Fig. 5.7). The switch from migratory to invasive phase induces expression of invasive surface proteins—circumsporozoite protein (CSP) and Thrombospondin-related adhesive protein (TRAP) (Dundas et al. 2019). Both CSP and TRAP interact with heparan sulfate (HS) molecules and stick to the hepatocytes. The binding of CSP to HS has been proven by treatment of hepatocytes with heparinase enzyme (Vaughan et al. 2008; Keitany et al. 2016). The CSP-HS interaction has been exploited for development of malaria vaccine RTS, S/AS01; however, the vaccine was not found to be long-lasting in Phase 3 clinical trials. In hepatocytes, the sporozoites replicate, and transform into merozoites, which is the asexual infective form that targets erythrocytes (Soulard et al. 2015). The replication and maturation of *P. falciparum* merozoites take 10–12 days (Keitany et al. 2016).

5.6.1.2 *Plasmodium* Interactions in Blood Stages

Plasmodium in the merozoite stage acquires a pear-shaped structure which contains two unique organelles—the apicoplast (nonphotosynthetic plastid), and apical complex structures (rhoptries and micronemes). *Plasmodium* interaction at the blood stage can be divided into two phases: invasion/exit and cytoadherence. *Plasmodium*

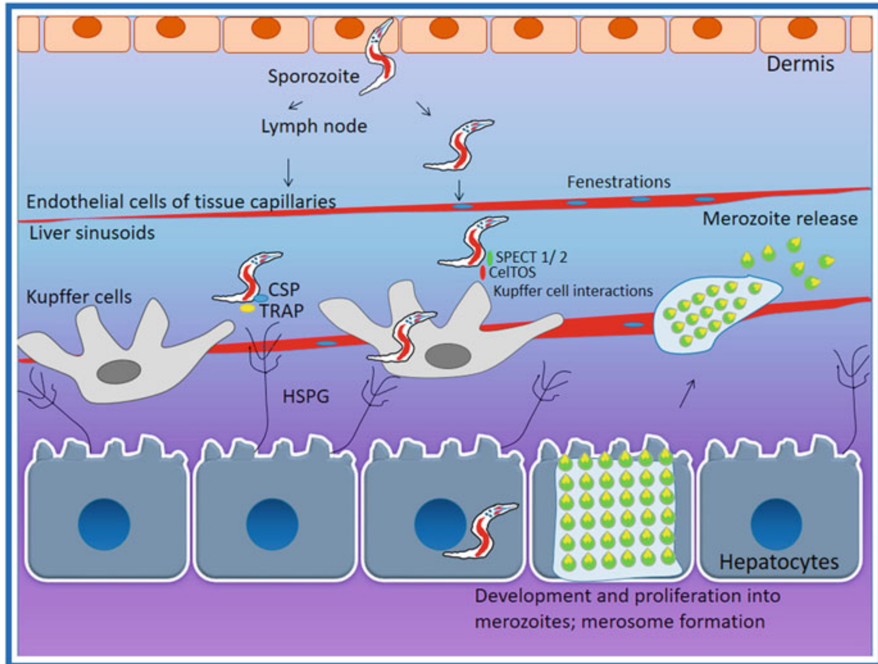

Fig. 5.7 Schematic showing the interaction of Plasmodium with host tissue and various proteins involved in the process. (Adapted from Acharya et al. 2017)

interaction in the blood stages involves numerous proteins that exhibit high affinity toward the erythrocyte membrane constituents. A brief description of the parasitic proteins and their mechanism of interaction with erythrocytes during invasion/exit of merozoites and cytoadherence are discussed in the following paragraphs.

Invasion/Exit of Merozoites

Interaction of RBCs with merozoites is mediated either by sialic acid (SA)-dependent pathway or sialic acid-independent pathway (Ord et al. 2012). The SA-dependent pathway involves expression of EBL proteins (Erythrocyte-binding ligands) and PfRH1 protein in merozoites. The EBLs show great affinity toward sialylated proteins called glycophorins. Glycophorin A, B, and C have been shown to bind EBL-175, EBL-1, and EBL-140 respectively (Gaur and Chitnis 2011). Special vacuolar apical structure, micronemes act as storage unit for EBL protein. Except PfRH1 protein, all the other PfRHs are members of SA-independent pathway together with MSP (merozoite surface protein). MSP is a surface protein that interacts with nonglycosylated regions of an anion transport protein, Band 3 (Baldwin et al. 2015). The interaction results in phosphorylation of Band 3 and its dissociation from ankyrin and spectrin. The dissociation leads to cytoskeletal rearrangement in erythrocytes, and formation of a vacuolar structure in which the parasite survives (Baldwin et al. 2015; Acharya et al. 2017). Once inside the

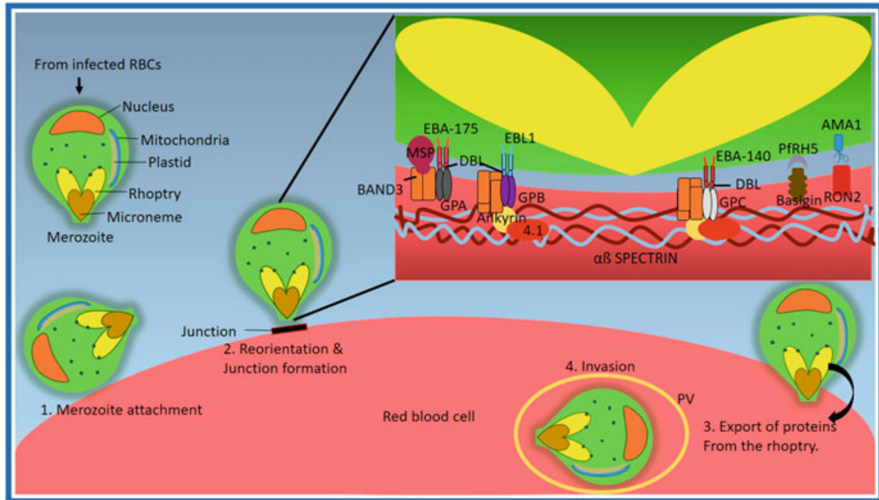

Fig. 5.8 Schematic representation of various RBC-associated proteins involved in interaction with the merozoite stage of *Plasmodium* infection. (Adapted from Acharya et al. 2017)

erythrocyte, merozoite phosphatase catalyzes dephosphorylation of Band 3 and restoration of cytoskeleton. PfRHs except PfRH1 have been documented as binding partners of complement receptor 1 (PfRH4), Basigin (PfRh5) (Triglia et al. 2011; Aniweh et al. 2020; Volz et al. 2016). PfRH5 also elicits expression of antibodies having inhibitory functions against merozoite invasion in humans. Due to their potent relevance in invasion, MSP and PfRH5 have been pursued extensively for development of antimalarial vaccines (Fig. 5.8).

Other *Plasmodium* factors such as apical membrane antigen (AMA1), rhoptry neck protein (RON), CD55, DARC, and PLP2 have been shown as important triggers of invasion. Similar to EBLs, AMA1 is also localized in micronemes and transported to the merozoite surface after invasion (Singh et al. 2010). While RON is localized in rhoptries and gets embedded into RBC membrane after secretion. The RON and AMA1 interact with each other and behave as a connecting junction between RBC and merozoites (Srinivasan et al. 2011). The RON–AMA1 interaction is a key step in the invasion process and has been targeted for development of antimalarial drugs. Srinivasan et al. reported three small molecules namely, 7-cyclopentyl-5-(4phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine, liarozole hydrochloride, dimetacrine as potent interactors of AMA1 and inhibitors of RON-AMA1 protein–protein interaction (Srinivasan et al. 2013). Further, two RBC surface receptors, CD55 and DARC (Duffy antigen receptor for chemokines) also play a significant role in *Plasmodium* attachment and invasion (Scully et al. 2019). Moreover, a recent in vitro study of PLP2 protein showed that PLP2 enhances the permeability of endothelial cells of vascular system by increasing the intracellular Ca^{2+} levels causing necrosis of endothelial cells of hepatic blood vessels and blood–brain barrier (Shivappagowdar et al. 2021). Inside the erythrocytes, merozoites

divide mitotically and disseminate through rupturing RBCs (Acharya et al. 2017). The exit of merozoites from RBCs is guided by parasitic proteases such as falcipain-2, PfSEA-1 (*P. falciparum* schizont egress antigen1), and plasmepsins. Falcipain-2 and plasmepsins are cysteine and aspartate protease, respectively. They are involved in degradation of hemoglobin, ankyrin, and spectrin (Mishra et al. 2019; Rosenthal 2011). While PfSEA-1 is a parasitic antigen which is important for merozoite replication as well as exit from RBCs (Shivappagowdar et al. 2021).

Cytoadherence of Merozoites

Upon invasion, the merozoites proliferate in the encapsulated vacuolar structure called parasitophorous vacuole (Acharya et al. 2017). As RBCs are devoid of organelles, the parasite establishes a membranous network called Maurer's cleft for communication with the extracellular environment (Chakravorty et al. 2008). The Maurer's cleft acts as a postal system for transport of parasitic proteins into RBC compartment and extracellular environment. The transport route is aided with *Plasmodium*-specific signal sequence and chaperones. The *P. falciparum* erythrocyte membrane protein family (PfEMP) is one of the well-documented members of the Maurer's cleft. PfEMPs are surface antigens having a hypervariable extracellular domain (HVD), a transmembrane domain, and an acidic intracellular domain (Smith et al. 2013). The HVD exhibits differential affinities toward a wide array of host receptors. PfEMPs are a part of a large macromolecular complex known as knobs situated on an infected RBC surface. Other than PfEMPs, knobs are made up of RESA (Ring-infected erythrocyte surface antigen), MESA (Mature parasite-infected ESA), and KAHRP (Knob-associated histidine-rich protein) (Lee et al. 2019). Knobs are important protein complexes for cytoadherence of *Plasmodium* to infected erythrocytes (Ganguly et al. 2015).

5.7 PPIs in Prion Diseases

Prions are small intrinsically disordered proteins that prompt abnormal folding of cranial proteins (Scheckel and Aguzzi 2018). Prions are related to various diseases in animals as well as humans. They are transmitted in humans via infected meat products. The onset of prion-associated disease or TSEs relates to the conformational change in native cellular prion protein (PrPC) into a misfolded scrapie-like prion protein (PrPSc) (Chen and Dong 2016). Due to the misfolded state, PrPScs accumulate in the central nervous system and stimulate neuronal degeneration and vacuolation. Human prion diseases are neurodegenerative disorders known as transmissible spongiform encephalopathies (TSEs). The TSEs include fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS), Kuru, and Creutzfeldt-Jakob disease (CJD). Creutzfeldt-Jakob disease is one of the most common prion diseases in humans (Baldwin and Correll 2019). Cases of CJD can be sporadic, genetic, and acquired. Most of the cases recorded in humans are sporadic (85%); however, the pathogenesis of sporadic CJD (sCJD) is still not known (Kovacs and Budka 2008). The genetic CJD and acquired CJD (variant CJD and iatrogenic CJD)

accounts for 10% and 2–5% of CJD cases. Variant and iatrogenic CJDs (vCJD and iCJD) are caused by external prions, while genetic CJD (gCJD) is caused due to genetic and/or epigenetic alterations of PrPCs (Houston and Andréoletti 2019). At present, no therapeutic or preventive treatments are available for prion disease, especially for vCJD and iCJD. Hence proactive surveillance of dietary intake of animal-based products is conducted for prevention of prion diseases (Houston and Andréoletti 2019). CJD is an implication of genetic alterations and M/V129 polymorphism in PrPCs. These alterations and polymorphic structure are widely utilized as biomarkers for detection of CJD in patients.

5.7.1 Conformational Features of Prion Proteins

Prion or PrPCs are cellular glycoproteins and consist of misfolded isoform (PrPSc) derived via post-translational modification in prion protein. The native structure of PrPC has been elucidated by nuclear magnetic spectroscopy as well as X-ray crystallography (Kovacs and Budka 2008). The mature PrPC is formed after splicing of N-terminal signal peptide and C-terminal peptide after addition of glycosylphosphatidylinositol anchor. PrPc in its native state is made up of 100 amino acids in both N-terminal and C-terminal regions. The N-terminal is unstructured, while the C-terminal is folded into three α helix and a short β sheet. The structured region of PrPC is stabilized by a disulfide bond between second and third helices (Estibeiro 1996; Bratosiewicz-Wasik et al. 2004). Glycosylation is a principal factor in structural integrity of PrPCs. Therefore, two Arg-linked glycosylation sites are present in the PrPC structure. Moreover, five repeats of an octapeptide region and a copper ion-binding site are present at the N-terminal region of PrPC (Wulf et al. 2017). The octapeptide-repeat region has been linked to inherited prion disease, following expansion due to frameshift mutations. Further, strong binding affinity toward Cu^{2+} ions has been elucidated in earlier studies suggesting PrPC's role in copper metabolism and transport (Collinge 2001; Mead et al. 2019). The conformational stability of PrPCs has been remarkably explained in a study conducted by Hosszu et al. describing the population distribution of folded and unfolded states of prion protein through HDX exchange experiments. The study suggested that PrPScs are formed from a kinetic folding intermediate and the unfolded state of the protein (Hosszu et al. 2005). Mutation in PrPC promotes interaction of PrPC and PrPSc, thus increasing the propensity of aggregate formation. Moreover, it has been observed that presence of PrPSc acts as seeds for conversion of PrPC to PrPSc (Singh et al. 2017).

PrPSc structure exhibits vast differences from the PrPC structure (Kanyo et al. 1999). The crystal structure of PrPSc has not been solved yet; however, based on FTIR and CD spectroscopy techniques, a model of PrPSc has been elucidated (Wille and Requena 2018). In contrast to PrPC, PrPSc is rich in β-sheets, and is covalently indifferent from PrPC. During conversion of PrPC to PrPSc, a crucial switch that is α-helix to β-sheets is observed (Elfrink et al. 2008). In recent years, the propensity of α-helices to form β-sheets have been studied extensively, and the observations are a

bit controversial (Wulf et al. 2017). For instance, Eghain et al. performed NMR conformational studies on α-helix-specific antibody-bound prion protein and found that the first helix (H1) is highly prone to β-sheet formation as compared to second (H2) and third (H3) helices (Eghiaian et al. 2004). Meanwhile Thirumalai et al. and Chakroun et al. reported that H2 and H3 exhibit the propensity to convert into β-sheets rather than H1 through molecular dynamic simulations. The β-sheet-rich region is known to play a vital role in the protein oligomerization process. In addition, PrPSc is insoluble in detergent, protease-resistant, and accumulates in the brain according to a unique pattern (Dima and Thirumalai 2004; Chakroun et al. 2010). The exact molecular process involved in conversion of PrPC to PrPSc is not confirmed yet, although several structural models have been hypothesized over the years. The most acceptable and the coherent model states that PrPC fluctuates between its native and nonfunctional intermediate states which aggregate together to form PrPSc (Wulf et al. 2017). The initial PrPSc formed acts as seed and catalyzes the PrPSc formation. The formation of PrPSc is affected by PrPC concentration, PrPC-PrPSc equilibrium, and the degree of complementarity between aggregating surfaces of prion protein (Wulf et al. 2017).

PrPC has been designated with several functions; however, no consensus over the role of PrPC in homeostasis has been concluded. Over the years, countless in vivo studies on mouse models have been conducted to understand the function of PrPC. PrPC is highly expressed in neuron and glial cells of the central nervous system. The nerve PrPC is compartmentalized in pre- and postsynaptic vesicles. This compartmentalization is believed to be involved in maintaining the synaptic structure by monitoring plasticity and synaptic transmission. The synaptic dysfunction is also an early event in prion diseases (Senatore et al. 2013). Moreover, feeble GABA-mediated synaptic transmission and long-term potentiation (plasticity) in hippocampal Schaffer collaterals have been observed in PrPC-deficient mice (Caiati 2012). Role of PrPC in sleep homeostasis has also been proposed which is also one of the prominent symptoms of prion diseases. PrPC has been proposed to be involved in regulation of circadian rhythms, slow wave activity (SWA), and sleep fragmentation (SF) (Wulf et al. 2017). ZH1, Edbg, and Prnp gene knockout mice models have been studied to identify the function of PrPC in sleep deprivation (Sánchez-Alavez et al. 2007). From the studies, altered circadian rhythms and increased SWA and SF were recorded in absence of PrPC while upon reintroduction of PrPC expression reversed the sleep deprivation in mice models (Tobler et al. 1996). The molecular basis of sleep regulation has been linked to calcium-dependent potassium channels, voltage-gated channels, and NMDA receptors. PrPCs are known to be functional in maintenance of myelin homeostasis with the help of GPCR receptor—Gpr126. As it is a glycoprotein, it is also believed to be involved in cell surface adhesion. Several proteins such as NCAM1 and laminin receptors have been recognized as PrPC-interacting molecules; however, the importance of interaction in vivo has not been identified yet (Mantuano et al. 2020). Other than NMDA receptors, PrPC has been identified as molecular interaction partners of several neurological receptors and proteins imparting a variety of functions (Wulf et al. 2017).

5.8 Conclusion

The impact of pathogenic infections on humans is as significant as lifestyle disorders. Their property of transmission, high proliferation rate, and facultative approach of survival are a concern worldwide. Moreover, evolutionary adaptations and high mutation rates give pathogens an edge for their survival in environment. Over the years, several epidemics and pandemics have been linked with such pathogens. The pathogenicity of a foreign entity is enormously affected by host–pathogen interactions. The pathogenic strains thrive at their best to enter the host system and proliferate with extreme prejudice. In this process, either pathogens get eliminated by the defense mechanisms or they find a suitable bypass. The tussle for survival is intricately guided through a variety of protein–protein interaction between host and the pathogen. Similar to any other condition, PPIs involved in host–pathogen interactions are key factors in deciphering the molecular mechanisms, therapeutic strategies against infections. Further, increase in antimicrobial-resistant strains due to usage of broad-spectrum antibiotics, drugs is another alarming threat to human population. Hence, unraveling the intricacies of host–pathogen interactions and their structural aspects boosts the generation of antimicrobial compounds, vaccines, and a highly specific approach in combating with such infections.

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Drug Design Methods to Regulate Protein–Protein Interactions

6

6.1 Introduction

Proteins actively participate in numerous cellular processes including transporting molecules, cell signaling, and catalyzing reactions' immune responses to a variety of pathogens. All the biological activities that are critical for an organism's health like DNA replication, transcription, translation, cell signaling are regulated by the formation of functional protein complexes (Ferrari et al. 2013; Rual et al. 2005; Stelzl et al. 2005). The protein complexes rely on the highly controlled protein–protein interactions (PPIs). These interactions among numerous proteins in the cell form a complex network of proteins, which is known as "interactome." This interactome plays a crucial role in both physiological and pathological processes. Any aberration in interaction of proteins can lead to the numerous diseases like neurodegenerative diseases, cancer, and other infectious diseases (White et al. 2008; Blazer and Neubig 2009; Rosell and Fernández-Recio 2018). Hence, PPIs have emerged as promising new drug targets in contrast to the enzymes, receptors which act as the typical drug targets owing to their key roles, and sophisticated regulation and specificity (Arkin and Whitty 2009; Loregian and Palù 2005; Nero et al. 2014; Milroy et al. 2014; Hill et al. 2014; Nevola and Giralt 2015; Jaiswal et al. 2019). Recently, PPIs have received great attention as therapeutics (Shin et al. 2017; Scott et al. 2016; Lu et al. 2020; Rudolph et al. 2021; Rodrigues et al. 2022) to treat numerous diseases including cancer, HIV, diabetes, asthma, arthritis, cardiovascular disease, lupus nephritis, etc. (Albach et al. 2018; Bailey et al. 2010; Rosell and Fernández-Recio 2018; Korycka-Wolowiec et al. 2019; De Weger et al. 2014; Arnhold et al. 2018; Holzer et al. 2015; Sun et al. 2014; Lehmann et al. 2016).

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6.1.1 Challenges to Modulate PPIs

Although, PPIs serve as promising drug targets, the modulation of PPIs by small-drug molecules is quite a daunting task. This is due to several challenges faced while designing the modulators for a specific PPI. These include: (1) the interface area for the interaction of proteins is 1500–3000 $Å^2$ which is quite larger as compared to the area of interaction between the receptor and ligand (300–1000 $Å^2$) (Smith and Gestwicki 2012; Cheng et al. 2007), (2) interfacial region between the proteins is highly hydrophobic in nature, (3) flatness of the interface with few pockets, grooves, clefts, or indents also poses a problem for the binding of small-drug molecules (Díaz-Eufracio et al. 2018; Arkin and Wells 2004; Buchwald 2010); (4) amino acid residues involved in interaction between proteins are contagious or noncontagious on the respective protein structures and thereby resulting in high-affinity interactions between the proteins, thus making it difficult for the small-drug molecules to interfere/dissociate such strongly interacting protein complexes; (5) in contrast to typical drug targets like receptors or enzymes, PPIs are deficient in presence of small endogenous ligands that can be used as reference for drug designing (Ivanov et al. 2013); (6) drugs that act on PPIs are high in molecular weight (>400 kDa) as compared to traditional small-molecule drugs (200–500 kDa), thus limiting them to follow the classical Lipinski's (rule of five) criteria (Shangary and Wang 2009; Buchwald 2010). Despite of these challenges, variety of low-molecular weight molecules targeting the large protein partners have been designed (Ivanov et al. 2013; Shangary and Wang 2009; Li and Xiao 2009; Shaginian et al. 2009; Toogood 2002; Cochran 2001; Gadek and Nicholas 2003; Stites 1997; Arkin and Wells 2004).

6.2 Role of Hot Spots in PPIs

Hot spots in PPIs refer to those amino acids that play an imperative role in interfacial regions of PPIs. These are the residues that make the highest contribution to the binding-free energy (Geppert et al. 2011; Moreira et al. 2007; Janin et al. 2008). Designing inhibitors for PPIs is cumbersome owing to the large interface of PPIs. However, the emergence of hot spot residues in the interface facilitated an easy roadway for designing the drugs specifically targeting PPIs (Shangary and Wang 2009). Number of hot spots in the PPIs increase as the area of PPI interface increases. Usually, the area surrounding the hot spots is 600 $Å^2$. Amino acids which act as hot spots are recognized by simple point mutation experiments. Specific amino acid residue is mutated to alanine, and then the difference between the binding-free energies is monitored to calculate its contribution to binding-free energy. Residue which results in an incrementation in binding-free energy by 2 kcal/mol upon mutation by alanine is regarded as a hot spot (Thorn and Bogan 2001). In comparison to the other amino acids, tyrosine, tryptophan, and arginine are majorly reported as hot spots in PPI interfaces (Moreira et al. 2007; Scott et al. 2016). Hence, these residues are mostly employed for designing drugs for inhibition of PPIs. Therefore,

despite of larger interface of PPIs, drugs are needed to focus only on hot spots to halt PPIs.

6.3 Identification of Druggable Hot Spots in PPIs

Several computational methods have been developed to identify druggable hot spots that constitute the binding interface in the specific PPI. Some of these methods including, CASTp, FTMaP, FTFleX, LIGSITE, Q-siteFinder, ANCHOR, pyDock, and Pocket Query are discussed below.

6.3.1 CASTp

CASTp refers to "computed atlas of surface topography of proteins," which is an online tool (http://cast.engr.uic.edu) that aids in locating the specific pockets, cavities, or voids on the protein structure. CASTp contains annotated details attained from PDB (protein data bank), OMIM (online Mendelian inheritance in man), and Swiss-Prot, about the specific residues belonging to protein structures. These annotations are employed to map voids, pockets, and other functional regions in protein structures. The server exploits semi-global pair-wise alignment methodology in order to attain mapping between the entries in OMIM, Swiss-Prot, and PDB entries. The updated current version of CASTp is being utilized to determine functional regions in the protein and also to determine the role of important residues of protein (Dundas et al. 2006).

6.3.2 FTMaP

FTMaP is a webserver (http://ftmap.bu.edu/login.php) that computationally scans the protein surface to probe the binding sites on the protein structure. This server identifies druggable sites on the basis of multiple solvent crystal structure methodology. Computational solvent mapping is carried out to predict the hot spots at the interface of PPI. The protein surface is mapped using the set of 16 organic small-molecule probes that exhibit differential chemical features. The probes also vary in their size and shape. Fast Fourier transform (FFT) is employed to scan the organic probes over both translational and rotational grid defined on the protein surface. Each receptor protein-probe complex is evaluated by utilizing energy function based on electrostatic and Van der Waals interactions. Regions where most of the clusters of probe bind are named as consensus sites (Kozakov et al. 2015a). The site which is populated with highest number of probe clusters is known as the main hot spot. The rest of the consensus sites are known as secondary hot spots. FTMaP is a reproducible method and is in concurrent with X-ray-based experimental methods. This computational solvent mapping methodology has been tested on 15 PPIs to identify druggable target sites in them (Kozakov et al. 2011).

6.3.3 FTFlex

FTFlex is an extended version of FTMaP and is freely available at http://ftflex.bu.edu. FTFlex enables the prediction of protein–protein binding site by taking into consideration both the side chain flexibility and conformational changes that occurs during the interaction among the proteins. Hence, FTFlex yields improvised outcomes in contrast to FTMaP. FTFlex scans those residues which are flexible within the binding site (Grove et al. 2013).

6.3.4 LIGSITE

LIGSITE program designed to determine the pockets or cavities in the proteins. Additionally, this program also allows the graphical display of those cavities in the protein. LIGSITE does not require any prior knowledge about the pockets or cavities in the protein. This algorithm can be viewed as an extension of pocket algorithm designed by Levitt and Banaszak. LIGSITE is an automated method that employs series of operations on cubic grid to identify cavities in the proteins in an efficient manner and in a reasonable time. The main advantage of this method is its high speed, as it takes around 5–20 s to find ligand sites on medium-sized proteins. Hence, this method can be used in comparative studies that involve large set of proteins for the scanning of pockets. Proteins contained in PDB are scanned for pockets by LIGSITE and the information about the identified pockets is stored in RELIBase database (Hendlich et al. 1997).

6.3.5 Q-Site Finder

Q-site finder identifies the ligand-binding site on the protein using differential approach. It takes into account an energetic criterion to define a binding cleft or a pocket on the protein. Q-site finder calculates the Van der Waals interaction energies between the protein and a methyl probe. Probes that exhibit favorable energies of interaction are clustered together, and their ranking is carried out in accordance with the total interaction energies. Clusters with most favorable interaction energy are assigned the first rank. Q-site finder has been designed to aid in accurately locating ligand-binding site for drug designing processes including both de novo designing and virtual screening. This method is also being employed to determine the functional site on the protein.

The success rate of identifying the binding site using Q-site finder is higher than that of Pocket-finder which is one of the commonly employed detection algorithm. Q-site finder is two times more effective in accurately mapping coordinates of ligands onto the predicted sites than Pocket-finder. One of the major advantages of this methodology is that it is capable of identifying relatively small sites. These sites have a volume roughly similar to that of the ligand, and are independent of the volume of the protein (Laurie and Jackson 2005).

6.3.6 ANCHOR

ANCHOR is an online tool that allows users to explore the protein–protein interface to identify the site suitable for small-molecule drugs. This tool employs the anchor residues that correspond to those amino acid side chains that are deeply or highly buried in the interfacial regions in PPI. These anchor residues are supposed to be the possible druggable pockets that can serve as major targets of small molecules. When a user provides protein-protein complex as a query to ANCHOR, it calculates the change in the solvent accessible surface area for every side chain upon binding the partner protein and its contribution to the binding-free energy. ANCHOR allows the interactive visualization of selected anchor residues in the pockets, and also permits to determine the stereochemical properties of the surrounding regions including charge–charge interactions and hydrogen bonding via Jmol-based tool. ANCHOR also includes the precomputed anchor residues retrieved from more than 30,000 entries of proteins contained in PDB having a minimum of two peptide chains. The anchor database can be queried using variety of keywords including amino acids, energy, buried region in order to assess the PPIs that are suitable drug targets for small molecules. Web server of ANCHOR and its database are free to access at http://structure.pitt.edu/anchor (Meireles et al. 2010).

6.3.7 Pocket Query

Pocket query is an online server developed by David Koes to identify hot spots, anchor residues, and also the hot regions at the interfacial zones of PPIs. Screening and sorting of such residues are performed on the basis of solvent accessible surface area, conservation of sequences, and scores based on energy values (Koes et al. 2012).

6.3.8 pyDock

pyDock is an energy-based docking as well as scoring program that enables the identification of hot spots without requiring the structures of protein complexes. This method is based on a novel strategy to determine the inhibitory sites to block the PPIs. Strategy employed in this program is based on merging molecular dynamics to generate transient cavities and docking-based interface hot spot prediction in order to choose the cavities suitable for drug targets. pyDock approach has been tested on set of data for which the comprehensive structural data of the protein–protein complexes along with their inhibitor are known. It has been observed that molecular dynamics via local conformational sampling generated the transient cavities that were analogous to the binding sites of known inhibitors and docking simulations were able to precisely reveal the best cavities (Rosell and Fernandez-Recio 2020).

6.4 Mechanism of Action of PPI Modulators

Protein–Protein interactions can be modulated in a variety of ways. The most common classes of PPI modulators are (1) PPI disruptors and (2) PPI stabilizers. Modulators which disrupt the PPI are known as PPI disruptors and the molecules that stabilize the PPI are known as PPI stabilizers. Both these types of modulators have been further classified into orthosteric and allosteric modulators based on their binding sites. An orthosteric modulator binds at the PPI interface. In contrast, an allosteric modulator binds at the site which is located remotely from the PPI interface (Fig. 6.1). An orthosteric disruptor disrupts the formation of protein complex by

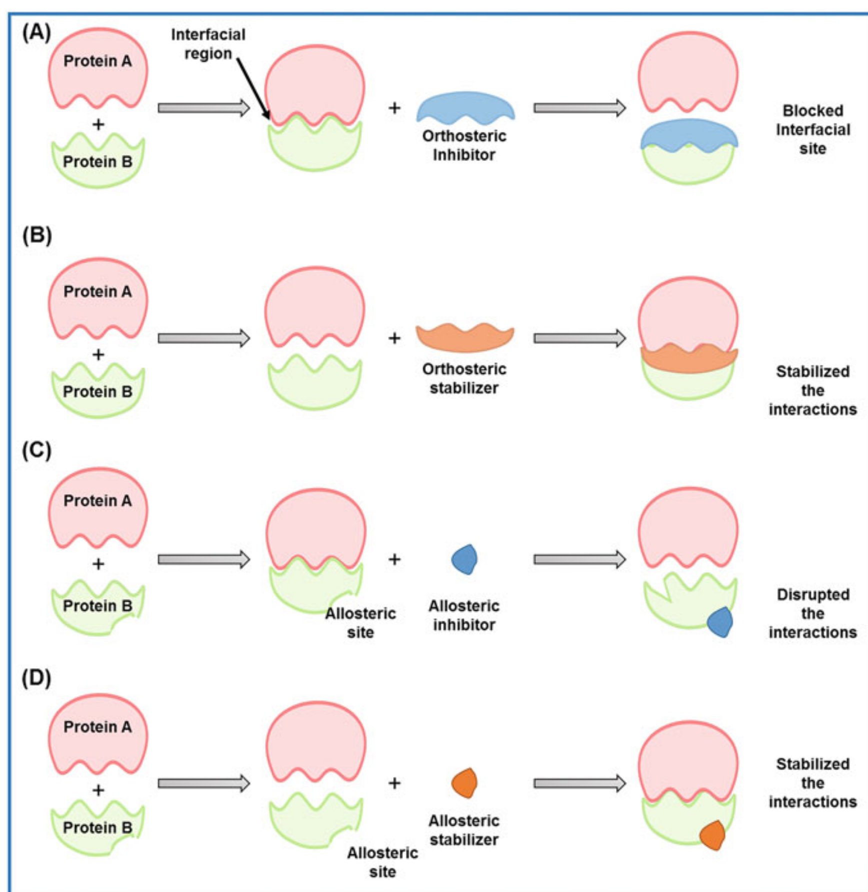

Fig. 6.1 Schematic representation depicting different mechanisms of PPI modulation. PPI modulators are of four types namely, (**a**) orthosteric disruptor, (**b**) orthosteric stabilizer, (**c**) allosteric inhibitor, and (**d**) allosteric stabilizers. If the modulator inhibits or stabilizes the PPI through interacting with the active site of the PPI partners, they are categorized as orthosteric. While the PPI modulators are known to be allosteric if they bind to the noncatalytic site of PPI partner to induce change in protein–protein interactions

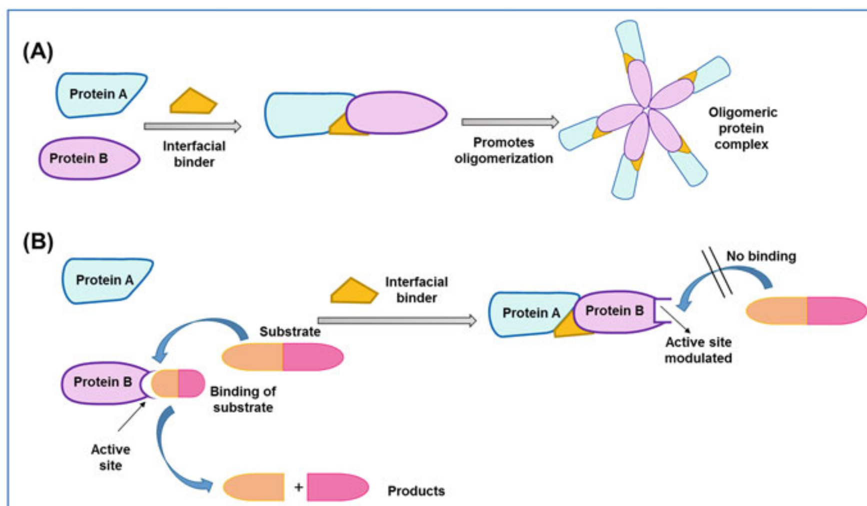

Fig. 6.2 Pictorial illustration of varied functional properties of modulators upon their interaction with PPI protein partners. (**a**) Illustrating the creation of a variant oligomer upon interaction of interfacial binder with the protein-protein complex. (**b**) Depicting the allosteric inhibition of a protein complex induced due to the presence of interfacial binder at the active site

directly competing with one of binding protein partners (Fig. 6.1a). Majority of modulators that are in clinical trial for targeting PPIs belong to the orthosteric disruptor class (Flygare et al. 2012; Souers et al. 2013; Xiong et al. 2014; Wang et al. 2014; Vu et al. 2013; Ding et al. 2013; Zhong et al. 2012). An orthosteric stabilizer stabilizes the PPI by binding at the newly formed site at the PPI interface (Fig. 6.1b). Such a site mostly occurs at the rim of interface, which is formed upon the interaction of two binding protein partners. Such orthosteric stabilizers upon binding to the PPI interface can also increase the stability of PPI by increasing the number of interaction sites between the binding proteins. Similarly, an allosteric inhibitor inhibits the formation of protein complex by binding to the site located away from the interfacial region of proteins (Fig. 6.1c). This approach is quite effective in cases where PPI interface is devoid of deep cavities. In contrast, an allosteric stabilizer aids in the interaction of protein partners via allosteric affect (Fig. 6.1d). Binding of an allosteric stabilizer can result in conformational change in one of the proteins, thereby enhancing its affinity for its binding protein partner (Fischer et al. 2015).

Interfacial binders are not only limited toward modulating the binding affinity of protein partners. Interestingly, in some cases, these binders occupy pockets created by homo-oligomerization or hetero-oligomerization of proteins to transform the dynamics of constituting proteins of the protein complex. This in turn results in hampering the functional properties of the complex-like oligomerization state (Fig. 6.2a) (Kessl et al. 2012; Tsiang et al. 2012; Sharma et al. 2014),

Table 6.1 List of some approved and under clinical trials PPI modulators involved in treatment of diseases

PPI target	Modulator	Disease	Status	Reference
Bcl-2 family	Venetoclax	Cancer	Approved	Deeks (2016)
c-Myc/Max	Nadroparin	Cardiovascular	Approved	Zhang et al. (2016)
CCR5/gp120	Maraviroc	HIV	Approved	Perry (2010)
IL-2/IL2-R	Apremilast	Psoriatic arthritis	Approved	Keating (2017)
Rac1	Azathioprine	Asthma	Approved	Anstey and Lear (1998)
Cyclophilins	Cyclosporine	Graft rejection	Approved	Tedesco and Haragsim (2012)
Transthyretin	Diflunisal	Rheumatoid arthritis	Approved	Brogden et al. (1980)
Immunoglobulin	Tacrolimus	Immunosuppressor	Approved	Scott et al. (2003)
MDM2/p53	Idasanutlin	Acute myeloid leukemia	Phase III	Skalniak et al. (2018)
XIAP/caspase 9	ASTX-660	Lymphoma	Phase I/II	Zhao et al. (2021)
LFA-1/ICAM-1	Lifitegrast	Dry eye	Phase IV	Perez et al. (2016)
β-Catenin/CBP	RPI-724	Liver cirrhosis	Phase I/II	Jiang et al. (2018)
CD40/CD40L	ABBV-323	Ulcerative colitis	Phase II	Argiriadi et al. (2019)
PD-1/PD-L1	Opdivo	Nonsmall cell lung cancer	Approved	Raedler (2015)

channel-opening (Lee et al. 2014), and enzymatic activity (Hammoudeh et al. 2014) (Fig. 6.2b).

Variety of modulators targeting different PPIs have been designed, and some of them have already reached clinical trials (Table 6.1). Majority of these modulators belong to the class of PPI inhibitors in contrast to PPI stabilizers. The development of PPI inhibitors has gained more attention in the era of drug discovery than PPI stabilizers. However, few PPI stabilizers have also been approved as drugs by FDA (Table 6.1). These stabilizer molecules are highly selective in nature as compared to PPI inhibitors, as they need to bind to both the constituting proteins of a protein complex simultaneously. This implies that druggable pocket is a resultant of two interacting protein partners, in contrast, PPI inhibitors bind only to one of the protein partners (Rosell and Fernández-Recio 2018).

The major obstacles in development of PPI stabilizers as drug are (1) lack of knowledge about the mechanism of action of PPI stabilizers, and (2) poor chemical space for PPI stabilizers in small-molecule chemical libraries (Rosell and Fernández-Recio 2018). Many of the PPI stabilizers have been found serendipitously, and only few of them are the logical outcomes of the rational drug design. Although, identification of hot spots at the PPI interface is beneficial for the development of PPI stabilizers, the process still needs further endeavors from the drug designing community.

6.5 PPI Inhibition Approaches

Several differential strategies are being followed aiming to inhibit the targeted PPIs. Four major approaches include: (1) Small molecule, (2) peptide, (3) antibody, and (4) natural products (Bakail and Ochsenbein 2016; Zinzalla and Thurston 2009). Each of these approaches, and the pros and cons associated with them have been discussed in detail in the following subsections.

6.5.1 Small-Molecule Approach

In the past decades, small molecules have proved to be effective drug molecules targeting PPIs. Till date, small molecules have targeted more than 40 important PPIs and many are in preclinical trial stage (Bojadzic and Buchwald 2018; Arkin et al. 2014; Berg 2003; Wells and McClendon 2007; Buchwald 2010; Mullard 2012; Villoutreix et al. 2014; Song and Buchwald 2015). However, several challenges are being faced while designing small-molecule inhibitors targeting PPIs. *Firstly*, in order to inhibit a PPI, molecules need to cover the large interfacing region of PPI, which is of thousands of square angstroms. This imposes a big challenge for the small molecule to compete with PPI interface. Further, presence of diversified and multifaceted binding sites in PPI interface also makes it difficult to search for an efficient inhibitor (Yan et al. 2008). *Secondly*, high-throughput screening approaches like thermal shift assays (Zhang and Monsma 2010), FRET (Fluorescence Resonance Energy Transfer)/BRET (Bioluminescence Resonance Energy Transfer) (Mattheyses and Marcus 2015; Bacart et al. 2008), SPR (Surface Plasmon Resonance) (Neumann et al. 2007; Agarwal et al. 2021), Fluorescence polarization (Du 2015), ELISA (Enzyme-linked Immunosorbent Assay) (Weng and Zhao 2015), etc., are required to identify the hits. Moreover, these techniques also sometimes yield false-positive outcomes due to the aggregation of protein, oligomerization, or allosteric binding. Hence, other highly sophisticated biophysical techniques such as NMR spectroscopy, X-ray crystallography, isothermal titration calorimetry (ITC) have to be employed, from which the structural and thermodynamic details of binding can be acquired to validate the potential hits (Agarwal et al. 2021).

There was a rapid increase in the development of small-molecule PPI inhibitors in eighties, when there was an emergence of analyzing energetics of protein-binding interfaces. Advancement in alanine scanning technique to explore the hot spot residues essential for the interaction of binding partners has further given momentum to the development of small-molecule PPI inhibitors. An assumption was made, according to which the small molecules compete with a protein for binding to the hot spot residues present on the other binding protein partner. It is not essential for the small molecules to cover the whole interfacial surface to block the PPI. These hot spot residues are known as "***sticky zones***" that are involved in interaction with their natural protein binders, and are also susceptible to bind the small molecules (Thangudu et al. 2012; Kozakov et al. 2015a, b; Shoemaker et al. 2012; Haynes

et al. 1992). Scrutinizing these hot spots opened up the new avenues for rational designing of small-molecule inhibitors. In general, most of the hot spots are located at the middle of the binding interface covering small to medium-sized area. This feature allows the small molecules to target hot spots effectively. One remarkable illustration is the inhibitors targeting bromodomains (Haynes et al. 1992; Filippakopoulos et al. 2010; Zuber et al. 2011; Baud et al. 2014; Fu et al. 2015; Ycas et al. 2020; Chen et al. 2022). Bromodomains bind specifically to the acetylated lysine residues containing motifs in the protein. Acetylated lysine residues interact with specific residues in bromodomains and insert deep into the binding pocket. Several small-molecule inhibitors such as JQ1(Fu et al. 2015), I-BET151 (Piquereau et al. 2019), PROTAC (Qin et al. 2020; Martín-Acosta and Xiao 2021), and I-BET762 (Fiorentino et al. 2020) targeting bromodomain proteins have been designed and validated successfully (Schwalm and Knapp 2022).

In case of PPIs with large interfacial regions, hot spots are located far away from each other and exhibit differential chemical functionalities. Designing inhibitors for such hot spots is quite arduous. Hence, keeping in mind the challenges in fabricating small-molecule inhibitors to interrupt PPIs, various groups across the globe took an initiative to analyze the chemical properties of the inhibitors designed to target PPIs. At present, more than 40 PPIs have been targeted and several databases including 2P2I (Bourgeas et al. 2010; Basse et al. 2012), iPPI-DB (Labbé et al. 2013), TIMBAL (Higueruelo et al. 2009, 2013b) have been dedicated to store the information of the PPI modulators. PPI inhibitors share some common properties like, most of them are bigger in size and higher in molecular weight (>400 Da) as compared to the traditional drugs. PPI inhibitors contain large number of hydrogen bonds, and are more hydrophobic in nature and contain minimum ring structures (Villoutreix et al. 2014; Morelli et al. 2011; Koes and Camacho 2012a). These properties are distinct from the properties that are usually used to screen drug-like molecules. This implies that such properties of inhibitors designed for PPIs violate Lipinski rule as mentioned above (Lipinski et al. 1997). Such divergence necessitates the development of databases that are specifically dedicated toward storage details of synthesis procedure of complex and chemically diverse inhibitors for targeting PPIs. Various computational tools work in conjunction with experimental screens in order to accelerate the process of screening appropriate inhibitors of PPI. Such strategy is being employed in cases where no appropriate known inhibitor could be employed as an initial point in a structure-based approach for developing inhibitors against PPIs (Wang et al. 2000; Christ et al. 2010; Betzi et al. 2007).

An alternative approach known as fragment-based approach is also being employed in which, in contrast to selection of higher molecular weight ligand, a group of small molecules (fragments) with lower affinity to one of the binding protein partners are selected initially and an inhibitor is designed in stepwise fashion via structure-based strategy (Murray and Blundell 2010; Erlanson et al. 2004a; Hajduk et al. 1999). In this approach, the molecule initially selected, exhibits low-binding affinity and an optimization procedure is used in which the size of the molecule is expanded in stepwise manner and at the same time, its binding affinity is also optimized (Blundell et al. 2002).

6.5.2 Protein-Based Approach

This approach utilizes the whole protein to block specific PPI. This strategy of utilizing whole protein is being employed in cases where the interfacial region between the interacting proteins is large in size. Monoclonal antibodies are being exploited to compete with interacting proteins to form a complex with one of the protein partners. Current approach necessitates the humanization of immunoglobulins in order to circumvent any immune reaction. Advancement in the era of humanizing antibodies has led to an increase in the development of antibodies for their applications as therapeutic and diagnostic agents. Numerous antibody-based drugs have reached the clinical trials and some of them have already been approved by FDA (McCafferty 2010; Mullard 2013, 2014, 2015; Bhutani et al. 2021). Phage display method has been used to engineer the first inhibitory antibody (Huse et al. 1989). This methodology allows the user to engineer ample number of immunoglobulin domains with binding affinities greater than that observed in nature (Rader and Barbas III 1997). Several other approaches that are based on immunizing the humanized murine models have also emerged to design antibodies specific to their target proteins (McCafferty 2010). Nanobodies that are present in camelids are also being employed to generate simple and stable molecules that can target specific PPIs (Mujić-Delić et al. 2014; De Meyer et al. 2014). Nanobodies have proved to be robust in targeting G-protein-coupled receptors and are being employed as therapeutic and diagnostic agents (Mujić-Delić et al. 2014). Apart from immunoglobulin folds, other scaffolds such as Darpins (Kummer et al. 2012; Plückthun 2015), Alpharep (Guellouz et al. 2013; Urvoas et al. 2010), etc. are also being evolved to target PPIs. These scaffolds are characterized by the repetitive sequences and are also known as artificial antibodies. Engineering of such scaffolds have been upregulated with an advancement in the computational tools. These tools aid in modeling and designing specific protein-based modulators against specific PPIs with high accuracy (Whitehead et al. 2013). Amalgamation of such strategies along with phage display and deep sequencing techniques opened up new avenues for the generation of numerous highly specific protein binders (Fleishman et al. 2011; Correia et al. 2014; Jardine et al. 2013; Whitehead et al. 2013). In silico methods have also emerged that help in reducing the immunogenic profiles of designed inhibitory proteins (King et al. 2014).

Most of the natural or engineered protein-based inhibitors block the PPI by competing with one of the binding protein partners. This implies that these protein-based inhibitors mostly follow an orthosteric mechanism to block the interaction (Kim et al. 2012a). However, in a few cases they also accomplish their PPI-blocking actions by following an allosteric mechanism (Kim et al. 2012a). Major limitation of this approach is that these antibodies, nanobodies, and other protein-based inhibitors are large in size which pose difficulty in their penetration into the cell. Hence, such inhibitors are more specifically designed for the extracellular targets.

6.5.3 Peptide/Peptidomimetic-Based Approach

Peptides constitute the "more natural" alternate approach to block the interaction of proteins. Peptidomimetics, that refer to the synthetic molecules mimicking the secondary structure of proteins have also gained attention by pharmaceutical industries. Development of peptide-based therapeutics/peptidomimetics-targeting PPIs are associated with several advantages that include: (1) Peptides are highly flexible which implies their adaptability to interact with large interfacial regions; (2) Peptides are highly selectable and potent owing to the modularity in their structure; (3) Intermediate size (size more than small-molecule-based inhibitors, and less than protein-based inhibitors) of these peptides prevents their accumulation in tissues; and (4) Peptide-based inhibitors are highly biocompatible owing to their low toxicity levels (Higueruelo et al. 2013a; Mabonga and Kappo 2020; Datta et al. 2017). However, the development of peptide-based inhibitors is not devoid of few limitations which include: (1) More susceptibility of peptides for their degradation by proteolytic enzymes; (2) quick clearance of these peptides from the circulation; (3) peptides exhibit lower ability to cross physiological barriers; and (4) peptides exhibit lower immunogenicity.

Despite several limitations associated with peptides; considerable efforts have been made to eliminate the major bottlenecks that impair the development of peptides as PPI modulators. Attempts have been made to increase the permeability of peptides through cells and tissues. Cell-penetrating peptides (CPP), which contain a specific amino acid sequences make them capable of penetrating into the cells, are being conjugated to the peptides either at the N- or the C-terminus (Planel et al. 2010). Such a conjugation of CPP sequences to the targeted peptides increases their penetration capacity. An alternative approach is also being followed by the researchers across the globe to strengthen the cell-penetrating efficiency in the peptide inhibitors. This approach involves the modification of positively charged residues which are not involved in interaction with binding protein partner (Smith et al. 2008). Further, in order to improve the bioavailability of peptides and reduce their susceptibility to proteases, these peptides are subjected to some chemical modifications that include their conjugation with some smart-specific linker peptides. Several peptides have been designed and have been successfully employed to modulate PPIs. These peptides have been classified on the basis of the type of chemical modifications that are employed to fabricate them. Some of the significant classes of these chemically modified peptides are discussed in the following sections.

6.5.3.1 Cyclic Peptides

Cyclic peptides are being designed to block specifically interacting proteins. These cyclic peptides are the resultant of head to tail cyclization that aids in increasing their stability against physical denaturation and enzymatic degradation. Peptides fabricated in this manner also showed significant biological activity along with enhanced bioavailability. Numerous synthetic methods such as classical amide bond formation, imine-induced Ser/Thr ligation, addition of asparagine, etc. have

been developed for the cyclization of peptides (Tavassoli et al. 2008; Wong et al. 2013; White and Yudin 2011). Several cyclic peptides have been designed that were able to successfully block PPIs. For instance, cyclic pentapeptides, have shown to impair the viral budding mechanism, via blocking an interaction between host protein TSG101 and HIV Gag protein (Tavassoli et al. 2008). Recently, a molecule selected from the library of 64 million genetically encoded cyclic peptides was identified with an ability to block the dimerization of C-terminal-binding protein transcriptional repressor. This cyclic peptide has been employed to treat breast cancer cells. Treatment by these cyclic peptides showed reduction in fidelity and also a decline in the proliferation and colony-forming potencies of breast cancer cells (Birts et al. 2013).

Side chain groups are also being utilized to form cyclic peptides. For instance, the formation of disulfide bridges between the cysteine residues and the coupling reactions of differential nature have also been employed for the formation of cyclic peptides. Lactam-bridged peptides that are formed as a resultant of amide condensation between the side chain of lysine (K) and glutamic acid (E)/aspartic acid (D) are rigid in nature, also possess a well-defined α-helical structures, and improved PPI modulatory actions (Felix et al. 1988). Such cyclic peptides have tailored to bind third PDZ domain (PDZ3) of PSD-95, to block the interaction between NMDA receptor and PSD-95 (Li et al. 2004). Cyclization mediated by interaction between side chains is majorly focused to constraint the secondary structures of peptide, more specifically to promote the formation of α-helix.

6.5.3.2 α-Helix-Constrained Peptides: "Stapled" Peptides

Grubbs et al. developed a novel protocol for the synthesis of macrocyclic helical peptides via application of olefin metathesis reaction (Blackwell and Grubbs 1998). Later, Schafmeister et al. introduced α-helical conformation in small peptides by fabricating all hydrocarbon cross-linking in the peptides via metathesis reaction (Schafmeister et al. 2000) (Fig. 6.3a). Further, several research groups have employed the metathesis reaction to develop stapled α-helical peptides that have a potency to serve as a PPI modulator (Kim et al. 2011). These stapled peptides are highly stable, resistant to proteases, and exhibit great cellular uptake efficacy, and higher affinity for its substrate. Walensky et al. generated stapled peptides that can interact with BCL-2 family proteins which are the important regulatory players in the process of apoptosis. These stapled peptides are known as "stabilized alpha-helix of BCL-2 domains," and have shown to inhibit the progression of xenografts of human leukemia in vivo (Walensky et al. 2004). This stapling strategy has also been followed to design inhibitors against variety of interactions including p53-MDM2 (Brown et al. 2013; Chang et al. 2013; Bernal et al. 2007, 2010), MCL-1-NOXA (Stewart et al. 2010; Walensky et al. 2004), the MAML1–ICN1–CSL complex that belongs to NOTCH signaling pathway, etc. Stapled peptides have also been designed against specialized undruggable PPIs that involve extended and shallow interfacial regions for their interactions. For instance, GTPase signaling pathway has been regulated by designing a hydrocarbon-based stapled peptide-based inhibitor against Rab8a–Rab8a effector interaction (Spiegel et al. 2014). These stapled

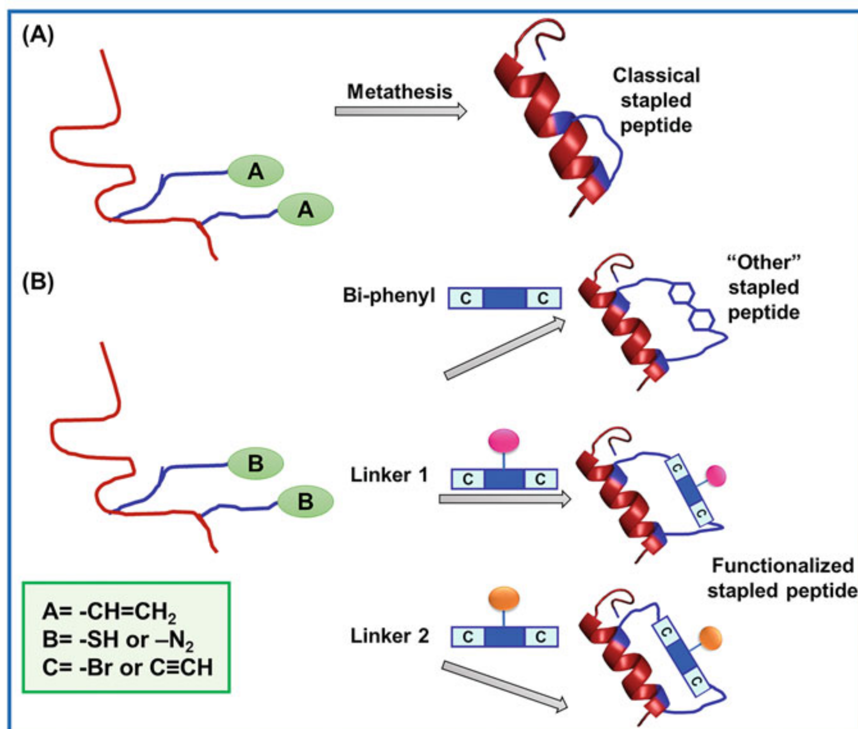

Fig. 6.3 Figure illustrating mechanisms of formation of different kinds of stapled peptides. The conversion of small peptides into α-helical conformation via metathesis reaction with the help of chemical groups such as: (**a**) hydrocarbons (allyl) form the classic stapled peptide. Several groups such as (**b**) sulfhydryl, nitrogen group are added to the classic-stapled peptides with the help of rigid linkers to obtain highly specific functionalized stapled peptides

peptides are also being used as protein probes for elucidating the structural properties of a protein and dissecting an interactome of a protein.

Apart from hydrocarbon-stapled α-helical peptides, several other methods have also evolved to induce helical formation in the small peptides (Fig. 6.3b). Rigid linkers are being employed that mediate the formation of helix via a reaction between a pair of cysteine residues present at desired location, mostly Cys residues at *i*th position and *i* + 7th/*i* + 4th/*i* + 11th in the series. Muppidi et al. utilized bisarylmethylene bromide as rigid cross-linker to link cysteine residues present at *i*th and *i* + 7th position. Cross-linked peptides showed improved cell permeability and an inhibitory action against p53–MDM2 interactions (Muppidi et al. 2011). Spokoyny et al. reported the discovery of perfluoro aromatic molecule as a cross-linker between the cysteine residues located at *i*th and *i* + 4th position in a small peptide. This cross-linker was designed to interact with C-terminal domain which belongs to HIV-1 capsid assembly polyprotein (Spokoyny et al. 2013). Further, click reaction that involved Cu-catalyzed alkyne-azide cycloaddition also aided in

inducing helical conformation in short peptides (Cantel et al. 2008). This strategy has been employed to fabricate inhibitors against interactions involving β-catenin and BCL9 (Kawamoto et al. 2012). Double click strategy has been applied in tailoring inhibitors against p53–MDM2 interaction which involved the reaction between diazidopeptides and dialkynyl linkers to generate bis-triazole-stapled peptides under catalysis by Cu(I) (Lau et al. 2014). A distinct approach that involves the introduction of HCM motif in the peptide also promotes α-helical content in the peptide. The presence of His at *i*th position and an attachment of artificial bidentate chelating group (for instance, 8-hydroxyquinoline) to the side chain of an amino acid present at $i + 7$th position has shown an increment in the helical content of the peptide owing to their metal (Zn^{2+}) coordination properties (Smith et al. 2013).

6.5.3.3 β-Hairpin-Stabilized Peptides

Various strategies have focused on designing and stabilizing β-hairpin peptides for the modulation of PPIs. β-hairpin, structurally comprises of two antiparallel β-strands connected via a loop or turn. Stabilized β-hairpin has been fabricated to mimic the helical epitope present at segment located at N-terminus of p53 protein. This peptide showed inhibitory action against p53–MDM2 interaction via binding to MDM2 with nanomolar affinity (Robinson 2013) (Fig. 6.4). Such stabilized peptides augmented the construction of aptides that are drafted via an inspiration of the structure of antibodies. Aptides are highly efficient in binding with several targets with nanomolar affinity and slow dissociation rates (Kim et al. 2012b). Aptides (APT) show the perturbation in the interaction between fibronectin extra domain B (EDB) and FN8 domain of fibronectin, which mediates the adhesion and movement of cells. APT unfolds the EDB domain and displace an intramolecular β-sheet with an intermolecular β-sheet, thus disrupting the interactions between EDB and FN8 domains of fibronectin.

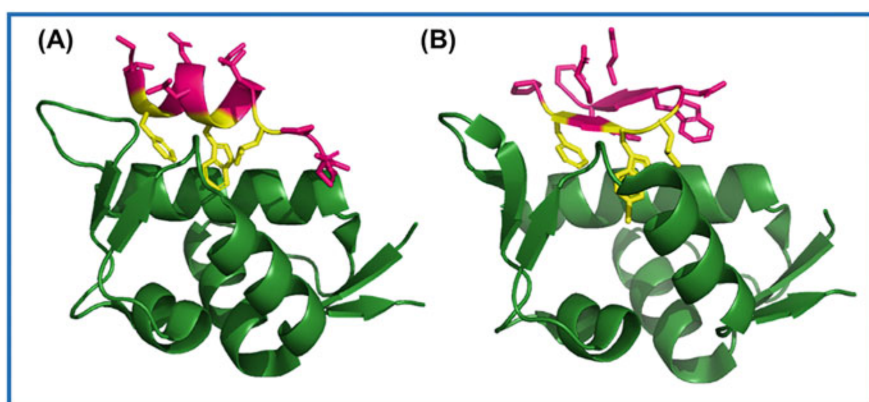

Fig. 6.4 Figure illustrates the interaction of p53-derived peptide with MDM2 protein. (**a**) α-Helical conformation (PDB: 3DAC), and (**b**) β-hairpin mimetics of the same α-helical conformation (PDB: 2AXI)

6.5.3.4 Bicyclic Peptides

Bicyclic peptides are rigidified PPI modulators with enhanced binding specificity, affinity, and stability. Bicyclic inhibitors were initially discovered via high-throughput screening of libraries created by phage or mRNA display. To further ensure the chemical diversity and improvement in bicyclic peptides, numerous synthetic means have been amalgamated with rational design approach. Lian et al. generated a bicyclic peptide by fusing a cyclic peptide with a cyclic cell-penetrating peptide (CPP). The resultant bicyclic peptide showed high-cell permeability retaining its specificity toward intracellular targets. This strategy has been applied to fabricate a bicyclic peptide in which CPP was fused to cyclic inhibitor of protein–tyrosine phosphatase 1B (PTP1B) (Lian et al. 2014). The formation of bicyclic peptide inculcated cell permeation ability in impermeable cyclic peptide inhibitor of PTP1B.

6.5.3.5 β-Peptides

β-Peptides, belong to the class of foldamers that are formed as a result of oligomerization of β-amino acids. These foldamers have an ability to form well-characterized secondary structures.

Seebach and Gardiner demonstrated that these β-peptides are distinct from α-peptides, as they contain an additional CH_2 group that is infused in each amino acid. CH_2 group can be added in two different positions, one is either between the Cα and nitrogen atoms, or secondly between Cα atom and the carbonyl group. β-Peptides as short as four amino acids long can also form secondary structures including helices, sheets, or turns. They exhibit highly stabilized structures which are resistant to metabolic and degradation-related enzymes. β-Peptides are being utilized to form mimetic of the epitopes of natural peptides involved in PPIs (Seebach and Gardiner 2008). Several research groups have worked in fabrication of β-peptides, mixture of α- and β-peptides, and have successfully employed them to target the interactions between p53 and MDM2 (Horne et al. 2008a, b; Denton et al. 2013; Bautista et al. 2010).

6.5.3.6 Peptoids

Peptoids are the outcomes of oligomerization of *N*-alkyl glycine, in which the attachment of side chain occurs at backbone nitrogen despite at α-carbon. Peptoids are highly resistant to proteases and possess an ability to acquire a predictable conformation (Bicker and Cobb 2020). Hara et al. designed effective peptoid-based inhibitors against P53-HDM2 complex which is devoid of any groups and promotes the formation of helix (Hara et al. 2006). Further, Murugan et al. fabricated cyclic peptomer inhibitors with the ability to interact with polo-box domain of polo-like kinase 1. Such peptomers are the outcomes of combination of peptoids and α-peptides (Murugan et al. 2013).

6.5.3.7 Miniproteins

Miniproteins refer to small proteins with enhanced stability and affinity. They are also capable of mimicking large interfacial surfaces of PPIs involving antibodies and

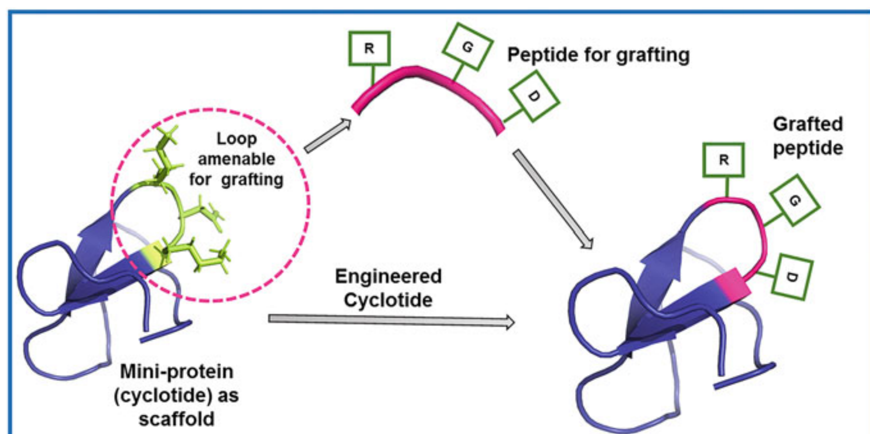

Fig. 6.5 Schematic representation of cyclotides (left), the plant-derived class of miniproteins. The cyclotides are used as PPI modulator either in its wild type state (cyclotide) or engineered by introduction of functional epitopes known as grafted peptides (right) for target-specific modulation of a PPI

protein receptors. DARPins, designed ankyrin repeat proteins, are antibody mimetics with an ability to mediate several PPIs (Poluri and Gulati 2017b; Gulati and Poluri 2019). These engineered mimetics with an enhanced specificity for protein binding are being employed as therapeutic agents in cancer. These small mimetics possessing potency similar to large proteins have geared up their usage in the field of tailoring PPI modulators (Boersma and Plückthun 2011). Cyclotides, represent another class of miniproteins derived from plants. They are being utilized as scaffolds for designing PPI modulators (Craik et al. 2007). An introduction of functional epitopes in cyclotides resulted in an additional class of peptides that are known as grafted peptides (Fig. 6.5). This strategy has been employed in designing an antagonist to block the intracellular degradation of p53 (Ji et al. 2013). Avian pancreatic polypeptide is another miniprotein scaffold in which an epitope to recognize MDM-2 was introduced (Kritzer et al. 2006). Further, inhibitors to block the androgen–estrogen receptor interaction have also been fabricated by reengineering and mutational strategies (Seoane et al. 2013). Miniprotein with high specificity to interact with membrane protein D114 has also been tailored to block the interaction of D114 with Notch1 receptor to abolish the vascularization of cancerous cells (Zoller et al. 2012).

6.5.3.8 Photo-Switchable Peptide Inhibitors

Photo-switchable peptide inhibitors are designed by introducing photosensitive cross-linkers in the peptide for regulating their structures and modulating their inhibitory actions (Schierling et al. 2010; Guerrero et al. 2005). Nevola et al. designed photo switchable inhibitors in order to regulate the process of clathrin-mediated endocytosis (CME). These peptide inhibitors were named as traffic light peptides which act as photoregulators of CME (Nevola et al. 2013).

6.5.3.9 Natural Products

Natural products serve as imperative templates for the designing of PPI modulators. To date, several natural products that function by targeting PPIs have been approved as drugs by FDA (Newman 2008, 2020; Rishton 2008; Newman and Cragg 2020). It has been presumed that number of these natural products does not obey Lipinski's rules and some of them also possess molecular weights more than 500 Da (Ganesan 2008). Further, Koehn along with his companions also found that although some natural products have not followed Lipinski's rule of five, they have been developed as orally active drugs (Zhang and Wilkinson 2007).

Modern drug discovery approaches are majorly interested in targeting signaling pathways, and the potencies of natural products are being exploited in this aspect. Natural products with highly diversified molecular framework provide an ideal initial point to construct the compound libraries for high-throughput screening. Natural products provide a scaffold that are amenable to chemical modification for generating highly variegated molecular structures. This enables the fabrication of libraries with highly diversified molecular structures (Milroy et al. 2007, 2008; Pascolutti and Quinn 2014; Newman and Cragg 2015; Kumar et al. 2021). Advancements in the novel chemical engineering techniques that assist in designing high-throughput compounds on the basis of structural framework of natural products have further accelerated the development of natural products-based chemical libraries (Arya et al. 2005; Morton et al. 2009). Progression in the era of recombinant DNA technology has further allowed the modifications of biosynthetic machinery at genetic level in plants, bacterial, viral, and fungal cells, thus enabling them to yield novel modified versions of natural products (Wilkinson and Micklefield 2007). Biological prevalidation of structures, binding efficacy, and specificity toward their target constitutes the major advantages of usage of natural products and their derivatives for drug designing. Hence, natural products serve as exquisite PPI modulators, with their three-dimensional structures and array of functional entities to differentiate between the highly related proteins (Whitty and Kumaravel 2006).

Till date, numerous natural products have been employed as PPI inhibitors. For instance, inhibitors against HIF-1 (Hypoxia-inducible factor-1) pathway have been designed on the basis of natural products obtained from natural sources. HIF-1 performs angiogenesis and glucose metabolism-related functions. HIF-1 also participates in growth and survivability of cancer cells (Lee et al. 2007; Zhou et al. 2006; Giaccia et al. 2003; Liao and Johnson 2007). Hence, researchers are targeting HIF-1 to block cancer-related pathological conditions. Several inhibitory compounds that have been discovered against HIF-1 are either the derivatives of natural products or are natural products themselves. HIF-1 is a heterodimer formed as a resultant of interaction between HIF-1β and HIF-1α subunits. The association between two subunits occurs via an interaction between PAS-A and PAS-B domains contained in the subunits. HIF-1 interacts with HRE (hypoxia response elements) in order to induce the expression of hypoxic response genes. Hence, it is essential to block the interaction between PAS domains of HIF-1β and HIF-1α subunits to suppress the functioning of HIF-1. Park et al. found the inhibitory action of rolitetracycline, a natural product, against the interaction between HIF-1β and

HIF-1α via an ELISA screening process (Park et al. 2006). Rolitetracycline was found inactive in cell-based assays, probably owing to its cell impermeability. However, its rigid structural framework is being utilized as a scaffold to design a library of cell permeable inhibitors (Zinzalla and Thurston 2009). Another natural product inhibitor known as chetomin was identified as a blocker of interaction between HIF-1α and its coactivator p300/CBP-binding protein. This interaction is essential to augment the transcription of HIF-1 (Kung et al. 2004). Chetomin was found to disrupt the tertiary structure of P300 via interactions with its CH1 domain. However, animal studies showed high toxicity of chetomin that limited its future development as drug. Nonetheless, the chemical structure of chetomin serves as a good initial point to generate chemically diversified libraries (Zinzalla and Thurston 2009). Structural variations and stereochemistry play an important role in protein-natural product interaction. For instance, several classes of plant secondary metabolites such as flavanols and flavonoids have been substantiated for their stereochemically induced anti-inflammatory efficacy. A structure-guided activity of flavanols and flavonoids against an inflammatory chemokine, CCL2 suggested an augmented inhibitory activity as a result of hydroxyl groups of annular ring-B and glycosidic ring respectively of plant metabolites (Joshi et al. 2020, 2021).

6.6 Approaches to Discover Modulators of PPIs

It is highly a daunting task to target PPIs owing to the unique characteristic features of their interfacial regions. Interface regions of PPIs are relatively large and flat in contrast to conventional protein targets which contain specific binding pockets. Therefore, designing and identifying effective leads to target PPIs using methodologies based on the concepts of classical chemistry are impotent. Hence, it is quintessential to establish innovative approaches to screen PPI modulators. In the past few decades, the area of fabricating PPI modulators has flourished with an immense development in the approaches to design and identify them. Four major approaches have evolved for the lead identification targeting PPIs which include: (1) high-throughput screening, (2) fragment-based approach, (3) structure-based approaches, and (4) virtual screening. Each of these methods have been described in detail in following sections.

6.6.1 High-Throughput Screening

High-throughput screening (HTS) is a traditional methodology employed to discover conventional drug targets. It has also gained importance in the era of development of drugs targeting PPIs. Identification of leads targeting PPIs using HTS, specifically targets hot spot residues in PPI interface. Compound libraries for HTS are developed mainly for conventional drug discovery that exhibits limited chemical diversity, thus excluding the chemical space of PPI inhibitors, thus necessitating the designing of libraries containing chemical space which is suitable for discovering PPI inhibitors.

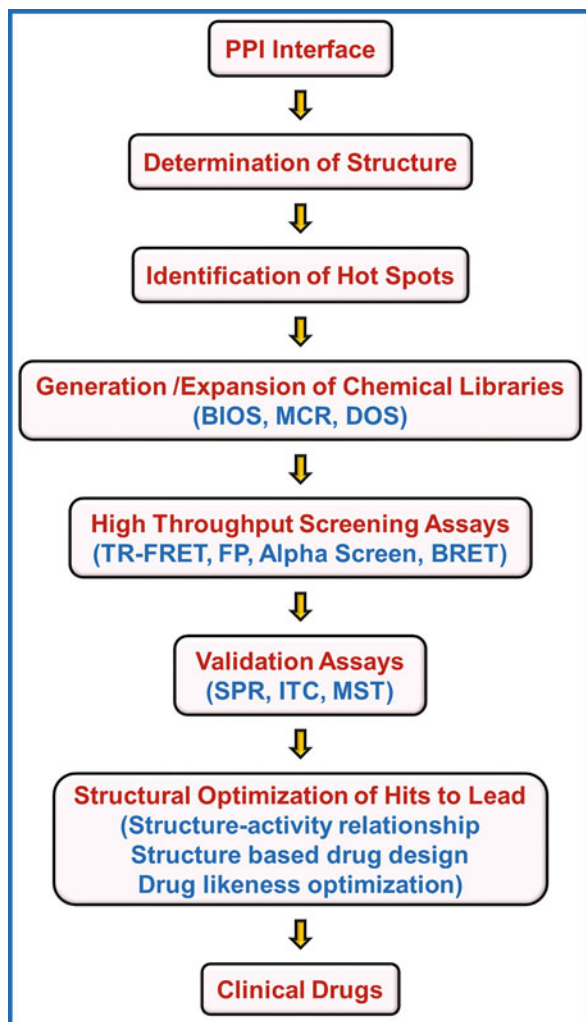

Fig. 6.6 Schematic representation of the workflow involved in the high-throughput screening of small-molecule inhibitors of PPIs

Moreover, large PPI interfaces result in low affinity of initial hits from HTS campaigns. Considering these aspects, compound screening is performed at high concentrations, which results in lesser number of hits and high false-positive outcomes. Flowchart describing the steps involved in HTS of small-molecule inhibitors for PPIs is shown in Fig. 6.6.

Success in the identification of PPI inhibitors is strongly dependent on the presence of suitable compound libraries and HTS-compatible assays. To date, several compound libraries have been developed including Enamine, Specs, Maybridge, ChemDiv, Chembridge, ZINC, etc. (Cheng et al. 2012). Apart from these libraries, several combinatorial chemical libraries have also been established by the constructive efforts of synthetic chemists. Several reliable and robust assays

including fluorescence resonance energy transfer (FRET), fluorescence polarization (FP), and amplified luminescent proximity homogeneous (Alpha Screen) have been applied successfully to screen appropriate inhibitors of PPIs (Agarwal et al. 2021). For validation of hits, low-throughput assays including isothermal titration (ITC), surface plasmon resonance (SPR), and microscale thermophoresis (MST) techniques are being employed. Binding parameters including dissociation constants, and binding kinetics (K_{on}/K_{off}) have been attained using these techniques, which play an important role in selecting hits for optimization of structures (Agarwal et al. 2021). Further, medicinal chemistry approaches including SBDD (structure-based drug design), SAR (structure-activity relationship), PK (pharmacokinetic), and pharmacodynamics (PD) are used to optimize the hits in analogous way as in the case of conventional drug targets. Success rate of HTS approach is largely dependent on the choice of size and chemical diversification of compound library, and on the target assay. It is worth noting that availability of structural details of PPI complex and the hot spots provides an invaluable contribution for the improvement in efficiency and success rate of drug discovery (Sheng et al. 2015). Presently, HTS approach is highly active in pharmaceutical industry, to screen initial hits targeting PPI. It is highly inspiring to note that numerous PPI inhibitors that are under clinical trial are the outcomes of HTS campaigns (Bojadzic et al. 2021; Shin et al. 2020; Makley and Gestwicki 2013; Zhuang and Sheng 2018).

6.6.1.1 Chemical Libraries for HTS

As discussed earlier, the success rate of HTS largely depends on the choice of an appropriate chemical library. High-molecular weight and complex structures of PPI inhibitors in contrast to traditional drug molecules demand expanded compound libraries. Such expanded compound libraries are generated by the expansion of present compound libraries to cover new chemical space that will allow better sampling of PPIs. Further, new compound libraries are also being generated that are more efficient and diverse for screening PPI inhibitors. With the advancement in the field of synthetic chemistry, focused compound libraries to scan targets for PPIs have been designed. Such libraries contain synthetic molecules which are outcomes of novel synthetic reactions including biology-oriented synthesis (BIOS), multicomponent reactions (MCRs), domino/cascade reactions, diversity-oriented synthesis, etc. (Sheng et al. 2015). Some of these compound libraries constructed for searching PPI inhibitors are discussed briefly in following sections.

Biology-Oriented Synthesis (BIOS)-Inspired Compound Library

Compound libraries constructed on the basis of natural products are enriched with PPI inhibitors. Based on this notion, goal of BIOS is to design compound libraries that contain natural products in which the biochemical activity along with the structural information is grafted (Wetzel et al. 2011; Bon and Waldmann 2010). Typically, a compound library generated on the basis of BIOS contains 200 to 500 compounds with a hit rate ranging from 0.2% to 1.5% (Wetzel et al. 2011).

Multicomponent Reaction (MCR)-Inspired Compound Library

MCR is being used at higher pace to accelerate the generation of compound libraries. MCR strategy exploits "one step, one pot" reaction in which two or more reactants react to yield one product (Ruijter et al. 2011). MCR-derived libraries have been exploited to screen inhibitors for variety of PPIs including p53/MDM2 (Antuch et al. 2006), HIV-1/gp414 (Xu et al. 2006), and Bcl2 (Antuch et al. 2006). Although, MCR-derived compound libraries are highly efficient, still these libraries are lagging behind owing to the lack of molecular and stereodiversity in the compounds contained in them (Ruijter et al. 2011). Dömling's group employed MCR-derived libraries to screen variety of inhibitors of PPIs. For instance, a MCR inhibitor derived from imidazole analog has been identified for Bcl-w/BH3 peptide interaction. Interestingly, this compound was also found to induce apoptosis in HL-60 leukemia cancer cell line.

Monfardini et al. have developed a novel combinatorial approach that couples various steps including anchor-based design, MCR-based generation of focused virtual libraries, virtual screening followed by chemical synthesis and biological screening to identify specific PPI inhibitors. ANCHOR simulates the hot spot residues present at the interfacial region of PPIs. An ANCHOR-based virtual library that involves the merits of both SBDD and HTS has been constructed with the aid of MCR. This strategy has been adopted to discover inhibitor against Bir3 domain of XIAP, which is an X-linked inhibitor of apoptosis (Monfardini et al. 2011).

MCR approach has also been employed in discovering inhibitors against p53–MDM2/MDM4 interactions (Koes et al. 2012; Schreiber 2009; Czarna et al. 2010; Boltjes et al. 2014). Trp23 is known as a hot spot residue in the interaction between p53 and MDM-2, hence, it had been employed as an anchor for the construction of virtual libraries. Initial screening of compounds via molecular docking approach is followed by the chemical synthesis of compounds with higher ranks. Further, biological assays depending on the modes of binding of compounds, their structural diversity, and synthetic feasibility assessments were performed to determine the final hits against PPIs (Czarna et al. 2010; Boltjes et al. 2014).

Diversity-Oriented Synthesis (DOS)-Derived Compound Library

DOS represents an efficient strategy for the simultaneous synthesis of more than one highly diverse compounds (O'Connor et al. 2012). The major goal of DOS programs is to synthesize libraries that are structurally and functionally more diverse to cover the chemical space including the new regions of the bioactive compounds. Success rate of DOS-based libraries relies on the generation of scaffold diversity within the library. Such libraries exhibit high stereochemical complexity levels, which are highly advantageous in scanning the compounds with highly selective-binding efficiencies (Clemons et al. 2010). Further, the preparation of complex compounds in DOS libraries involves no more than five synthetic steps. Hence, during the screening process, once hits have been attained, it is easy to synthesize the hit compounds in sufficient quantity. Thus, it will be easy to design focused libraries using the specific hit structure. This implies that DOS libraries affirm the selection of

suitable hits against PPIs, thereby gearing up the process of designing PPI inhibitors toward the complex road of drug designing (Nielsen and Schreiber 2008).

The construction of DOS-dependent macrocycle libraries has shown significant contribution toward the discovery of PPI inhibitors. Majorly the structural characteristics of compounds in macrocycle libraries are responsible for their potential to serve as PPI inhibitors (Marsault and Peterson 2011; Beckmann et al. 2013; Grossmann et al. 2014). Owing to the conformational preorganization of macrocycles and restriction in their conformational flexibility, such macrocycle compounds potentially serve as mimetics to interact directly with hot spots, thereby specifically binding to the topologically defined PPI surfaces. Additionally, such macrocyclic compounds possess better cell permeability in contrast to peptide-based PPI inhibitors. Nielsen et al. employed DOS library to scan inhibitors against the interaction between patched (Ptch 1) and sonic hedgehog (Shh) proteins, which belong to an imperative sonic hedgehog signaling pathway (Nielsen and Schreiber 2008; Rubin and de Sauvage 2006). Marcaurelle et al. also contributed to designing and synthesizing DOS library based on natural products. This library constitutes 15,000 compounds, and possesses four different kinds of chiral cysteine-inspired scaffolds (Marcaurelle et al. 2009). Series of Bcl-xL and Bcl-2 inhibitors were discovered upon the biological screening of the library designed by Marcaurelle (Marcaurelle et al. 2009).

Cascade Reactions-Derived Compound Library

Cascade reactions are highly effective to facilitate the congregation of diversified scaffold in single-pot way. Zhang et al. presented a novel approach in which researchers amalgamated two synthetic approaches namely, cascade organocatalysis and divergent synthesis for the creation of novel privileged substructure-based DOS library (Zhang et al. 2013b). Further, a contemporary-focused library was constructed that contained a new spiro-tetrahydrothiopyran-oxindole scaffold as a resultant of organocatalytic enantioselective Michael–Michael cascade reaction of motifs like oxindole and tetrahydro thiopyran (Zhang et al. 2015). Biological screening of such a library resulted in new types of inhibitors against p53–MDM2 interaction, that also showed antitumor activity (Wang et al. 2016).

6.6.1.2 Assays for HTS of PPI Inhibitors

Discovery of PPI inhibitors using HTS depends not only the libraries, but also on the choice of suitable assay which plays a crucial role in successful achievement of hits using HTS. Few important assays that are usually employed to discover PPI inhibitors include, time-resolved fluorescence resonance energy transfer (TR-FRET), fluorescence polarization (FP) assay, Alpha screen, bioluminescence resonance energy transfer (BRET).

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

FRET aims at recording the nonradiative transfer of energy from a donor fluorophore to an acceptor fluorophore. This transfer of energy occurs when both donor and acceptor are located close to each other. Hence, distance between the donor and

acceptor is a crucial factor for energy transfer. It has been shown that occurrence of transfer of energy takes place only in those cases in which the distance between a donor and acceptor lies in the range of 10–100 Å (Wu and Brand 1994). FRET is being commonly employed in high-throughput screening of PPI, peptide-binding, and enzymatic assays (Ma et al. 2014). In case of PPIs, the yellow fluorescence protein (YFP) and the cyan fluorescence protein (CFP) are mostly used as acceptor and donor fluorophores. Each of these fluorophores are fused with their binding protein partners (Schaap et al. 2013). The phenomenon of FRET is observed only in those cases where there is an interaction between the two fused proteins. When a small molecule blocks the interaction between the two proteins, a decrease in the transfer energy and an increase in the fluorescence intensity can be observed. However, this assay is associated with several limitations that include: (1) necessity to tag the proteins and peptides with donor and acceptor fluorophore for FRET, (2) low sensitivity in contrast to other fluorescence-based assays, since the autofluorescence background of the fluorophore disturbs FRET signal, hence it is quite cumbersome to differentiate between the signals due to weak interactions and background signal, (3) photobleaching during the course of time also greatly influencing the FRET signal, and (4) occasionally overlap of fluorescence emission pattern of small molecules with that of this common fluorophore donors. Hence, in order to address these issues associated with FRET, sophisticated techniques known as BRET and TR-FRET have evolved (Couturier and Deprez 2012; Degorce et al. 2009).

TR-FRET is time-resolved FRET which employs the usage of lanthanides including Terbium or Europium. Detection of signal from these lanthanides is simple, and easy to differentiate the signals as they arose as a result of autofluorescence from buffer, microplates, and small molecules due to the small fluorescence lifetimes of organic molecules. Excitation of Europium is carried out at 340 nm and its emission occurs at 615–620 nm. Further, this emission is transferred to excite far-red dye, for which the emission occurs at 665 nm (Owicki 2000; Degorce et al. 2009). Four most common TR-FRET assays including PerkinElmer (LANCE®), Cisbio, HTRF, and Life Technologies (LanthaScreen™) are commercially available. Small-molecule inhibitors against Myc/Max (Berg et al. 2002), Jun/Fos (Mathis 1995), 14-3-3/Bad (Du et al. 2013) have been identified using TR-FRET.

Bioluminescence resonance energy transfer (BRET) is also a FRET technique with a slight modification. BRET involves a bioluminescent enzyme as a donor, and the conversion of its substrate result into light emission. BRET possesses several benefits over other FRET-based technique including: (1) monochromatic light in FRET leads to complications in the signal, as in some cases it can excite both the donor and the acceptor simultaneously; (2) excitation light of FRET also leads to photo bleaching of the donor and autofluorescence of cell (Arai et al. 2001); (3) signal to noise ratio in BRET is ten times higher than FRET, thus enabling the user to use low concentration of protein samples (Couturier and Deprez 2012); (4) BRET requires simple and cost-effective instrumentation in contrast to FRET which requires a light source for excitation (Arai et al. 2001). BRET technology is

being employed to identify compounds that can act as inhibitors against TEM8/PA (Cryan et al. 2013), MDM2/p53 (Mazars and Fåhraeus 2010), YAP/TEAd (Guo and Teng 2015).

Fluorescence Polarization

Fluorescence Polarization (FP) is also well known as fluorescence anisotropy (Jameson and Ross 2010; Perrin 1926). The basic principle behind this technique is that polarized light is employed to excite a fluorescently labeled molecule, and the light emitted by the molecule with some degree of polarization which is inversely related to the molecular rotation rate. This property of fluorescence is being exploited to interrogate the interactions of small, labeled molecules with proteins. Further, FP assays are also being used to measure PPIs, DNA–DNA interactions, DNA–protein interactions. When the linearly polarized light enters the complex, depolarization of light occurs (Moerke 2009; Lodge et al. 2014). The extent of polarization of the emitted light is equivalent to the difference in the emitted light intensity with respect to that of excited light. Size of the fluorescently labeled molecule has high impact on the extent of depolarization of the initial polarized light that is used to excite the sample. Peptides are designed on the basis of hot spot residues involved in the interaction of proteins and are tagged with a fluorophore. In the absence of a small-molecule inhibitor, there is a formation of complex between a peptide and protein. This complex formation results in high FP value, then small-molecule inhibitors are added to the complex. If there exists a disruption of interaction between peptide and protein due to small-molecule inhibitor, fluorescently labeled peptide will be displaced and liberated from the protein-peptide complex which in turn results in low FP value. Low FP value is the resultant of more rotational mobility in the free peptide (Fig. 6.7). This technique is performed using 1536, 384 or 96-well plates. Data collection is carried out using fluorescence plate readers that are available commercially.

FP assay is ideal to screen PPI modulators as it only requires the synthesis of fluorescent-tagged peptide and its cognate-binding protein partner. FP assay is majorly employed for HTS, as it is cost-effective, simple and is devoid of any tedious washing steps to attain differential FP signals (Lea and Simeonov 2011; Owicki 2000). Additionally, FP assay requires only a small modification that involves fluorescent labeling of a peptide. However, this method is also not deprived of limitations such as: (1) The binding of fluorescently labeled peptide to protein should result in marked difference in the mass, which in turn can significantly affect the rotational mobility of the peptide resulting in considerable change in FP signal. It is endorsed that a difference of tenfold between the masses of protein and peptide is ideal to get an observable change in FP (Gribbon and Sewing 2003). (2) Existence of autofluorescence and light scattering affects the detection of signal. (3) It is highly challenging to identify false-positive outcomes when small molecules are used at higher concentrations. It is highly probable that aggregates of small molecules (at high concentration) interact with small peptides that result in aberrant signal (Shoichet 2006). It is interesting to note that FP assays have been favorably employed in investigating small-molecule inhibitors for several important PPIs

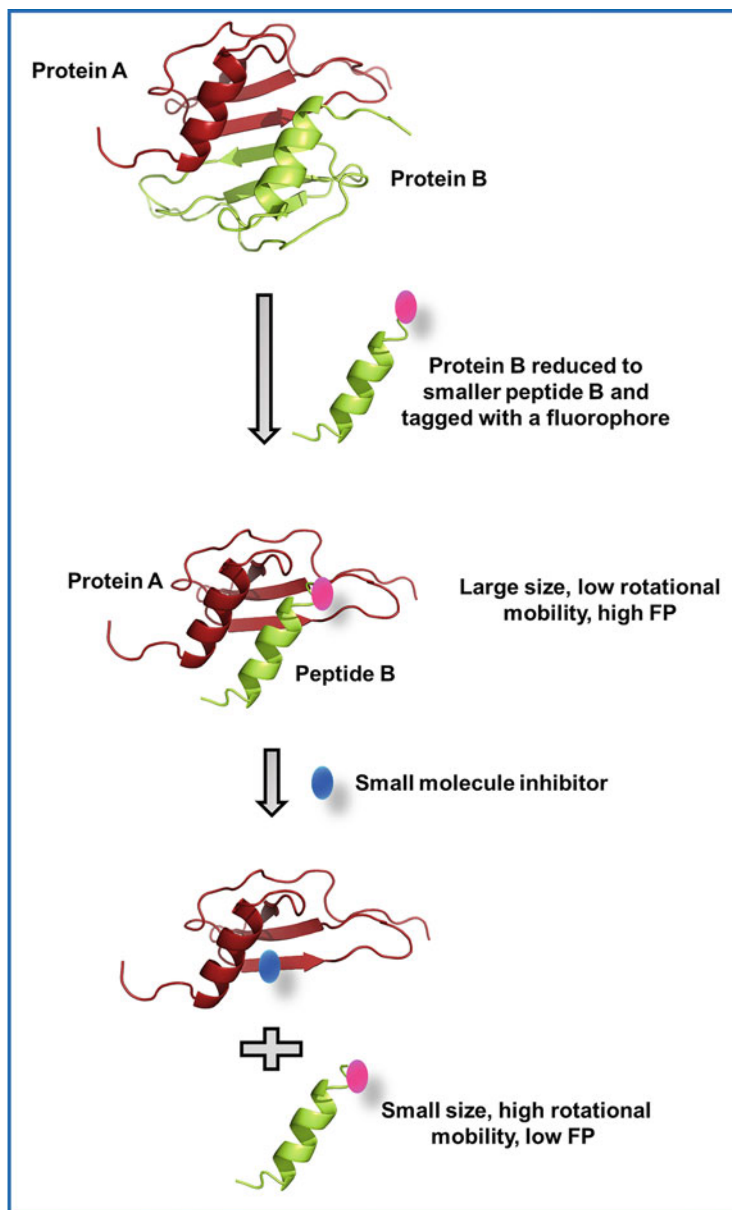

Fig. 6.7 Schematic illustration of detection of the inhibition of protein–protein interactions due to a small-molecule inhibitor with the help of fluorescence polarization (FP). In this method, a protein partner (protein B) is tagged with a fluorophore in order to detect the changes in rotational mobility of the tagged protein in bound and unbound state. In the bound state, due to the large size of the complex, a low rotational mobility or high fluorescence polarization (FP) is detected. However, in presence of an inhibitor, if the protein–protein interaction is disrupted, a low FP is detected due to small size and high rotational mobility of the tagged protein B

including c-Myc/Max (Kiessling et al. 2006), eIF4E/eIF4G (Moerke et al. 2007), Bcl-xL/Bak (Degterev et al. 2001), p53/MDM2 (Zhuang et al. 2011, 2012, 2014; Guo et al. 2012; Lu et al. 2006), and ZipA/FtsZ (Rush et al. 2005; Kenny et al. 2003).

Amplified Luminescent Proximity Homogeneous Assay (ALPHA) Screen

Amplified luminescent proximity homogeneous assay screen is also known as "alpha" screen, a technique used to measure the binding efficiency between the two interacting protein partners by utilizing donor and acceptor beads that are present in close vicinity to each other. These beads mediate an interaction between the proteins as they are coated by a hydrogel that offers functional groups for the bioconjugation of proteins. Donor beads are characterized by a photosensitive phthalocyanine, which when excited by laser at 680 nm, releases a reactive and an excited singlet oxygen. This reactive oxygen diffuses in solution within the distance of around 200 nm and transfers its energy to the acceptor bead which is tagged with a fluorescent or chemiluminescent group that results in a signal emission in the range of 520–620 nm. This technique is being widely applied in the field of identifying inhibitors for PPIs. For alpha screening, one of the binding protein partners is linked with the donor bead and other is linked with acceptor bead. For instance, a biotinylated protein is linked to streptavidin-coated donor beads, and a GST-fused protein is attached to anti-GST-conjugated acceptor beads. If these two proteins interact, the excitation of a donor by laser at 680 nm results in a signal. If a small-molecule inhibitor is disrupting the interaction between the two proteins, the excitation of donor will not be able to generate an emission signal. The major advantage of this assay over FRET is that it is able to investigate the signals from those interacting proteins which are present at distance (200 nm) higher than what is required for FRET assay. Further, alpha screen can be performed in a microplate which does not require filtration or washing steps (Zhuang et al. 2011; Guo et al. 2012; Lu et al. 2006). Other additional advantage of this system is that it involves nonradioactive beads. However, there are several limitations associated with this technique that includes: (1) requirement of specialized reader, as the signal is not detectable using luminometers and fluorimeters, (2) essential equilibration of the plate at room temperature as the signal is sensitive to temperature, (3) higher chances of observing false positives, as signal is obtained as a resultant of chemical reaction (Zimmermann et al. 2013).

Validation and Structural Optimization of Hits

High fraction of hits attained using HTS-targeting PPIs belongs to false-positive category. This is due to the differential nature of the assays, and due to their technical limitations and demerits. Hence, it is quite essential to perform a variety of orthogonal assays for studying PPIs for the authentication of the assays, and for the validations of hits against PPIs. Following the validation step, it is highly beneficial to perform the structural optimization of initial hits attained using HTS. This step may involve several minor modifications in the hits. However, such minor changes can significantly affect their binding affinities and specificities toward their target proteins. Structure-guided optimization of hits substantially increases their benign

interactions with hot spots present at interfacial regions of PPIs. This step is essential to transform the initial hits into the final leads (Zhuang and Sheng 2018). A variety of inhibitors have been screened using HTS, validated, and their structures have been optimized to convert them into effective leads against specific PPIs. For instance, discovery of benzodiazepinediones and nutlins against p53 and MDM2 interactions represents the most successful antitumor agents that were screened using HTS (Zimmermann et al. 2013). Waldmann's and his companions employed alpha screen HTS approach to identify the molecular inhibitors against PDEδ-KRAS (Zimmermann et al. 2013). Benzimidazole fragment 35 was identified as hit against PDEδ-KRAS, the fragment was able to bind PDEδ at its farnesyl-binding pocket with K_D value of 165 nM. Further, structure-based methods were exploited to optimize its structure and a dimeric compound 36 was obtained which showed increased binding affinity with K_D value of 39 nM (Zimmermann et al. 2014).

6.6.2 Fragment-Based Approach

Fragment-based discovery of drugs focuses on scanning molecular fragments against PPIs from the fragment-containing libraries. In contrast to HTS, fragment-based screening of compounds is superior in investigating the modulators of PPIs (Hajduk and Greer 2007). This is due to the fact that hot spots are present in discontinuous manner at PPI interface. Hence, it is easy to identify smaller fragments against the specific sites in the target, and then combining those smaller fragments to form lead fragments against the whole target. This is relatively an easier task in comparison to that involved in identifying larger hits at the initial stages, which is then followed by the optimization of those larger hits. Such an advantage of fragment-based approach is the major driver for the advancement in the fragment-based drug designing in pharmaceutical sector. Additionally, higher accessibility of small fragments in comparison to large compounds to smaller nooks in the proteins is highly beneficial. Further, higher solubility of such small fragments further adds an advantage to this approach. As small fragments are easy to synthesize, the libraries containing such small fragments can also be constructed and maintained with ease (Erlanson et al. 2004b).

Following the identification of fragment hits, fragment linking and optimization are carried out to attain final fragment leads against PPIs (Rees et al. 2004). Fragment linking and optimization further offer an advantage over large compounds since the process of linking small fragments and their optimization involves simple chemical pathways. In contrast, the optimization of large compounds involves the complete remodeling of the compound which is a quite cumbersome process. It is important to note that the affinity of binding of these fragments to the targets is low owing to their lower molecular weights and limited contact regions (Schuffenhauer et al. 2005). Hence, extremely sensitive assays have to be employed to identify the hits in these hits.

6.6.2.1 Identification of Fragment Hits

Identification of fragment hits is the foremost and highly challenging task. Continuous research efforts across the globe have led to the development of several methods to scan the fragment hits against the specific target PPIs. These methods have been divided into two categories namely: (a) nontethering, and (b) tethering methods.

Nontethering Methods

High-throughput screening usually employs the inhibitory assays, which implies that the binding of a lead to the protein will result in inhibitory effect to the interaction between the binding protein partners, and consequently affect their functionality. However, such HTS assays require the higher concentration of weakly binding compounds to screen them. Moreover, such assays are not feasible practically; since at higher concentrations, many of the compounds are insoluble and form large aggregates. Such assays are also more prone to false-positive outcomes. Hence to overcome these pitfalls, scientist moved in the direction of positive selection procedures for the identification of fragment hits (van Dongen et al. 2002). Three major techniques: (1) NMR spectroscopy, (2) X-ray crystallography, and (3) Mass spectrometry (MS) are being employed to positively screen the fragment hits (Agarwal et al. 2021). Of these three techniques, NMR-based screening of the fragments is the most extensively used approach for fragment screening. NMR can be carried out at high concentration and furnishes the ample information about the binding interactions between the fragment and target protein (Shuker et al. 1996; Liu et al. 2003). X-ray crystallography is also being employed by several research groups to dissect the interactions occurring between the small molecules/ fragments and target proteins. X-ray crystallography has also developed to decipher the fragment hits involved in interaction with proteins (Lesuisse et al. 2002; Lehn and Eliseev 2001; Jahnke et al. 2003; Fejzo et al. 1999, 2003; Blundell et al. 2002; Boehm et al. 2000). Combinatorial approach involving the usage of NMR spectroscopy and X-ray crystallography also has evolved to discover the fragment hits against specific PPIs. MS has also emerged to supplement positive selection of fragment hits; however, the information about the interactions attained using MS is limited in contrast to NMR and crystallography (Swayze et al. 2002; Kaur et al. 1997).

Tethering Methods

Nontethering methods discussed in the above section are based on the noncovalent interactions occurring between the small-molecular fragments and target proteins. Hence, all these methods require small-molecular fragments at higher concentration to investigate their binding with protein. In contrast, tethering methods are based on the reversible covalent bond formation between the target protein and fragment. Consequently, the interaction between the target protein and fragment can be investigated at lower concentrations of fragment. Tethering methodology also regulates the portion of the protein where the fragments can selectively bind. Additionally, lower concentrations (10–50 mg) of target protein enable the detection of more than 10,000 fragments. Major requirement for using tethering approach to

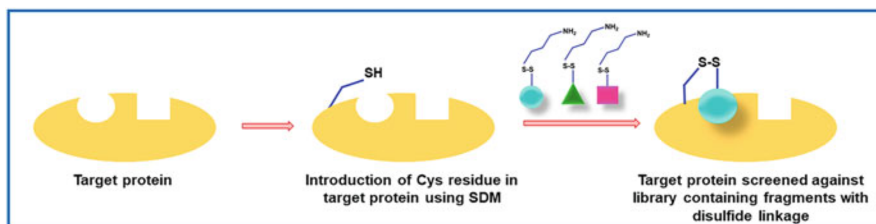

Fig. 6.8 Schematic representation of the basic tethering approach employed for disruption of protein–protein interaction and drug-discovery. In this approach, a target protein is introduced with a cysteine residue with the help of site-directed mutagenesis. The modified target protein is then used to screen inhibitor library containing sulfhydryl-containing fragments

determine the fragment hits against a specific PPI is the availability of coarse 3D model of target protein and its ability to be analyzed by MS (Erlanson et al. 2004b).

Basic protocol for tethering approach is depicted in Fig. 6.8. If a cysteine residue is contained in the target protein, then the target protein can be used in its original form. In case, target protein is not containing any cysteine residue, then a cysteine residue is introduced in the protein using site-directed mutagenesis. Position of cysteine residues should be in the vicinity (5–10 Å) of binding site or at the site of interest, and the cysteine residue should be exposed to the surface in order to expedite the thiol-disulfide exchange process. Following the fixation of position of cysteine residue in the target protein, the target protein is reacted with library of fragments containing disulfide linkages. This reaction is carried out in partially reducing conditions. Theoretically, cysteine contained in the protein will react and form disulfide bridges with the fragments. It needs a presumption that reactivity of disulfide in each fragment to the protein is equivalent, and there is no noncovalent interaction between the fragment and protein. Then at equilibrium conditions, there will be an equal proportion of protein disulfide linked each of the fragment contained in the library. However, there is shift in the equilibrium toward the particular protein-fragment complex, if there is a presence of the fragment in the library that has an inherent ability to bind to the protein at a site located in the vicinity of cysteine residue. This in turn can be utilized for the selection of a specific fragment against the protein. Reducing agents like 2-mercaptoethanol can be used in the reaction to tune the overall modification levels (Erlanson et al. 2000).

Several competing reactions can take place in a simple system containing two disulfide-containing fragments, a reductant, and a protein (Fig. 6.9). There is a possibility of attaining multiple equilibria and presence of multiple species. For instance, if the species of protein are considered, there will be the presence of four differential protein species including: (1) protein in Apo form, protein in complex with (2) reductant, (3) fragment A, (4) fragment B. Predominant species can be assessed using MS. If the molecular weights of each of the fragment are different, then it can also be easily determined that which fragment is predominantly interacting with the target protein. It has been shown that a compound can be selected from the library of 1000. However, it is highly convenient to screen ten

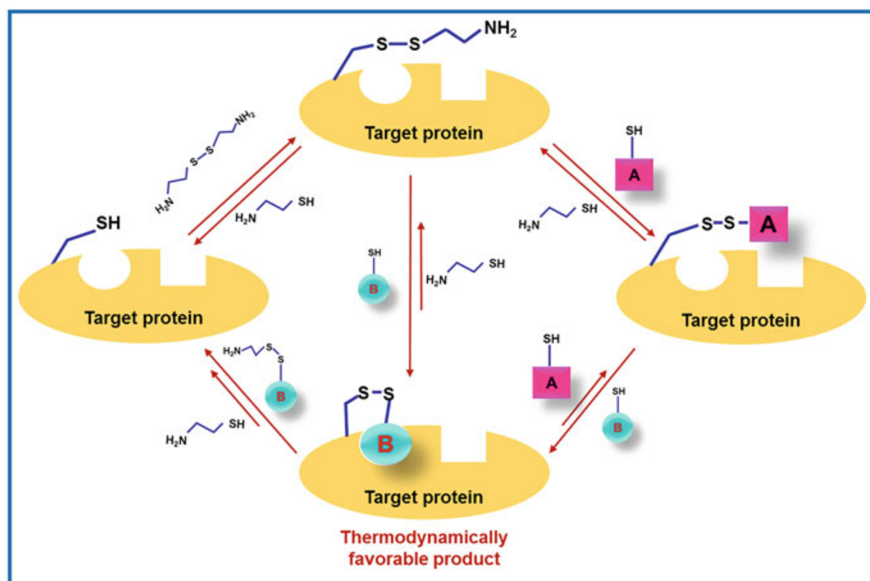

Fig. 6.9 Schematic representation of multiple reactions taking place in a system containing the target protein, multiple sulfhydryl-containing fragments, and a reductant. Even though due to presence of –SH group, a strong disulfide linkage can form between each fragment and the target protein, the fragment which has higher complementarity to the protein can only be able to form a thermodynamically favorable product

compounds in one shot, owing to the limitation with respect to the resolution in determining the high-molecular weights of proteins and smaller size of fragments and redundancy in the mass of fragments. In order to simplify the identification of fragments, it is suggested that the fragment libraries or pool of fragments should be constructed using the fragments which differ by a molecular weight of at least 5 Da from each other (Erlanson et al. 2004b).

There exists a close relation between tethering and dynamic combinatorial chemistry (DCC). DCC is a concept where there is an exploitation of reversible equilibria to select receptors or ligand from the pool of fragments (Ramström and Lehn 2002; Lehn and Eliseev 2001). This system relies on thermodynamically selecting the molecule which possesses higher affinity to bind to the target protein either through bond-forming or bond-breaking reactions in contrast to the other molecules contained in the library. There is a plausibility of several bond-forming reactions including oxime formation, metal–ligand interaction, boronic acid ester formation, imine formation, disulfide formation, etc., that can be utilized for tethering process. Among all these reactions, disulfide formation is the most ideal reaction for tethering, since the cysteine residues are naturally present in the protein, or it can be easily added into the protein using simple mutagenesis procedure. Additionally, the formation and breaking of disulfide bonds can be easily controlled using mild conditions, which does not adversely affect the properties of a biological molecule. Moreover,

the equilibria can also be shifted by altering the ratio of disulfide to free thiols (Gilbert 1995). The strategy of tethering using disulfide linkages has been employed by Obita et al. to scan the peptides against Tom20, a translocase protein (Obita et al. 2003).

6.6.2.2 Designing Fragments for Tethering

Ideally, the size of the fragments should be small for their employment in drug discovery process. They should possess molecular weight less than 250 Da. Further, these fragments should be highly functionalized and should not be accompanied with any toxicophores or associated with any other malignant functions. Feizo et al. and Jacoby et al. have discussed about various strategies of designing libraries for generation of leads in drug discovery (Jacoby et al. 2003; Fejzo et al. 1999).

Fragments used for tethering should possess thiol groups or disulfide linkages. Small molecules containing thiol groups are available commercially; however, as they are few in number, it is essential to build the customized fragment library. It is highly convenient if each molecule encompasses a distinct fragment on one side of the disulfide and a small solubilizing entity on the other side. For instance, aminoethanethiol is used as solubilizing group on the other side of disulfide bond. It is assumed that the reduction potency of disulfide moiety in each fragment is alike. However, in reality, the electronic and steric environment around disulfide has an impact on its overall stability. This is dependent on the group of atoms attached to the disulfide. These are the connective atoms that lie in between the disulfide and variable fragment region. In order to minimize the differential reactivities due to differential connective atoms, it is essential to employ same connective atoms between the disulfide and variable fragment region (Keire et al. 1992; Houk and Whitesides 1987).

Tethering with Breakaway Extenders

Tethering with breakaway extenders is an extension of tethering technique developed by Erlanson et al. to discover novel chemical inhibitors. This strategy involves the introduction of cysteine residue in the protein at a position that is located away from the active site. This cysteine residue is then modified using the cleavable extender. Upon cleavage of the extender, a thiol moiety will be exposed that lies in the vicinity of active site. Screening of libraries that encompass the fragments containing disulfides against such modified target protein will yield the fragments possessing inherently high affinity for the target protein, and in turn forming a disulfide linkage to the exposed thiol group (Fig. 6.10). This strategy has been applied to scan inhibitors for antidiabetic protein PTP 1B protein (Erlanson et al. 2003b).

Tethering with Extenders Used as a Fragment Assembly Tool

This is an additional tethering approach to identify the hits against the target protein. In this method, an extender is introduced into the target protein. Extender molecules should be characterized with following features including: (1) should have high inherent affinity of binding to the target protein; (2) propensity to interact with

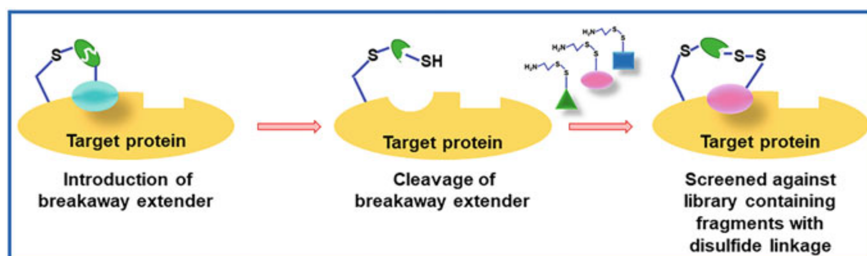

Fig. 6.10 Schematic representation depicting the screening of PPI inhibitors using tethering with the breakaway extender for identification of new fragments that shows affinity toward active site

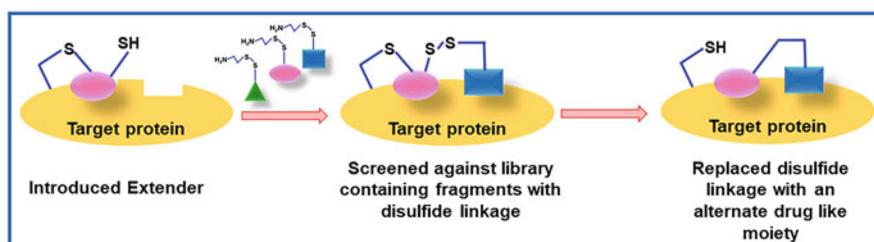

Fig. 6.11 Schematic representation screening of PPI inhibitors using tethering with extenders as a fragment assembly tool

specific cysteine residue contained in the target protein; and (3) possesses a masked thiol group. Fragment hits attained by initial tethering can be employed as extenders. The current approach employs the binding element of extender as the springboard in order to probe new fragments. Protein along with the extender is screened against disulfide-containing library of fragments to find out the fragment hits. These hits can able to interact with the protein, and also tend to form disulfide linkage to the extender. This approach yields the tight binding molecules comprising of the selected fragment linked with the binding element of the extender. The disulfide linkage can be replaced with a drug-like moiety possessing more stability in contrast to disulfide bridge (Fig. 6.11). This strategy of tethering with extenders has been used by Erlanson to design an inhibitor against caspase 3, which is an important mediator in the process of apoptosis. Inhibitor molecule scanned using this approach was able to successfully inhibit the apoptosis in cells (Erlanson et al. 2003a).

6.6.3 Structure-Based Approach

It is highly daunting task to design the PPI modulators in those cases where PPIs are devoid of any endogenous small molecule ligands. However, in such cases, hot spot residues play an imperative role in providing structural details and basis for fabricating PPI modulators. Currently, there are two structure-based approaches which are being used for tailoring PPI modulators. First approach is entirely on

the basis of structures of hot spots. De novo designing and bioisosterism are being adopted to attain novel small-molecule inhibitors against PPIs (Sheng et al. 2015). For instance, an amino acid, Hyp564 which plays an essential role in the interaction of VHL/HIF1α has been used in designing the inhibitor against VHL/HIF1α using de novo designing approach (Buckley et al. 2012). Second approach is the peptidomimetic approach which majorly employs proficiency in computational modeling and phage display. This approach works by simulating the secondary structures of crucial peptides involved in PPIs. Most of these crucial peptides possess α-helical structures; hence, the mimetics of such peptides are being designed to target PPIs (Mason 2010; Bullock et al. 2011). Several peptide inhibitors with α-helical structure have been fabricated against numerous PPIs including Bcl-2/Bax (Yin et al. 2005), C-Myc/Max (Yap et al. 2013), and MDM2/P53 (Chen et al. 2005). Two major structural-based approaches are discussed in detail in following sections.

6.6.3.1 Anchor-Based Approach

An anchor represents a hot spot residue present in one of the proteins involved in PPI. This protein is referred here as a donor protein and its partner protein is referred as acceptor protein. Anchor residue is highly crucial in molecular recognition and interaction with acceptor protein (Koes et al. 2012). Such residues are mostly located in the interior of the protein and mostly interact to the residues residing in the specific pocket on the surface of the acceptor protein. Hence, anchor residues are used as templates for designing their analogs and derivatives. In general, three strategies are being exploited for designing PPI inhibitors based on the anchor residue concept.

First method is based on scanning for the substructure of side chain of anchor residue to search for a new scaffold. This is followed by the improvement of its binding avidity to the acceptor proteins by extending the scaffold, so that it can interact with other hot spot regions in the acceptor protein. Second strategy is based on utilizing virtual library building tool or de novo designing software with an aim of extending the anchor itself to increase its interacting region with an acceptor protein. Third strategy is based on scanning for the bioisosteres of the anchor, which are imitating the crucial interactions occurring between the partner proteins. Bioisosteres used for fabricating small-molecule inhibitors are highly distinct in their structures in contrast to the anchor residues. Studies following such strategies for designing inhibitors based on structural information from anchor residues are discussed in the following subsections (Sheng et al. 2015).

Anchor-Based Designing of Inhibitors Against p53–MDM2 Interaction

p53–MDM2 interaction is majorly mediated by the hydrophobic interactions between the proteins. Hydrophobic residues including W23, F19, and L26 of p53 are involved in interactions with the small hydrophobic cleft located in MDM2. This hydrophobic cleft represents an ideal site to be used for designing inhibitors against p53–MDM2 interaction. W23 of p53 is the major interacting site, as the indole ring of W23 buries deeply into the cavity in MDM2 and its NH group is involved in hydrogen bonding with the carbonyl group in the backbone of MDM2 protein. Hence, researchers focused on W23 to search for the mimetics that can interact

with MDM2. Ding et al. found spirooxindole scaffold to mimic W23 using structure-based selection strategy and optimization tool (Ding et al. 2005). Spirooxindole p53-MDM2 inhibitor was able to bind to MDM2. Further, systemic optimization of spirooxindole resulted in MI-319 which is highly specific, orally active, and cell permeable inhibitor against p53/MDM2 interaction (Mohammad et al. 2009). Further optimization of this molecule yielded MI888 and its derivative MI-77301 which has reached to the phase I of clinical trial for its development as an anticancer agent (Zhao et al. 2015).

Czarna et al. have also utilized W23 as an initial point to build virtual libraries for screening of compounds targeting p53–MDM2. Multicomponent reactions (MCR) were used to generate the highly efficient libraries. Indole ring along with its bioisosteric derivatives including 4-chlororphenyl were embellished with differential functional entities and were subjected to MCRs. Screening of leads from the MCR scaffolds containing anchor residue was carried out using molecular docking. Compounds with highest ranks were selected based on their modes of binding, chemical diversity, and feasibility for synthesis. Selected compounds were synthesized and their binding avidities to MDM2 were determined using NMR spectroscopy. It was observed that seven compounds showed their binding affinity less than 60 μM (Czarna et al. 2010). Taking the advantage of strategy of using anchor residues, a novel web-based screening strategy known as anchor-based virtual MCRs has evolved, which is also known as "*Anchorquery.*" Anchorquery aids in designing virtually accessible synthetic compounds. Screening efficiency has been improved by Anchorquery as it takes into account the advantage of similarity search, pharmacophore, and docking-based tool kits. Anchorquery can be used to design modulators for the broad category of PPIs for which the structure and the information with respect to the anchor/hot spot residues are available. Anchorquery is a freely accessible tool to the users, and is available at http://anchorqueryccbb.pitt.edu (Koes et al. 2012).

Anchor-Based De Novo Designing of Inhibitors Against VHL/HIF1α Interaction
The interaction of hypoxia-inducible factor (HIF) with von HIPPEL-Lindau tumor suppressor protein (pVHL) plays an important role in regulating the cellular processes in response to changes in the availability of oxygen. pVHL interacts with HIF only when the proline at 564 position is hydroxylated, which is dependent on the oxygen levels. Hence, hydroxyproline 564 (Hyp 564) plays an essential role in the interaction of HIF with VHL (Min et al. 2002). Dennis et al. exploited Hyp564 as an initial point for the rational designing of inhibitors against VHL/HIF1α (Buckley et al. 2012). Hyp analogs were designed using a de novo designing software known as BOMB (Jorgensen 2009). A compound named as 82 displayed moderate binding avidity to VHL with an IC_{50} values of 117 μM. Further, SAR studies of molecule 83 on the basis of crystal structure of complex of ligand-VHL have resulted in next-generation small-molecule inhibitor 84 that binds with a sub-micromolar affinity.

Anchor-Based Bioisostere Replacement-Dependent Designing of Inhibitors Against β-Catenin–TCF Interaction

Yu et al. developed a hot spot-based strategy that employs bioisostere replacement for the rational designing of nonpeptidic small-molecule inhibitors against PPIs. They employed this strategy to design inhibitors against β-catenin-T cell factor (TCF) interaction. It was observed that lysine residues located at positions 435 and 508 in β-catenin play an imperative in interaction with TCF. Hence, novel fragments that are synonymous to the binding elements were designed using bioisostere library. An inhibitor with molecular weight of 230 showed K_d value of 0.53 μM for binding to β-catenin. This inhibitor showed Ki value of 3.14 μM for disrupting the interaction between β-catenin and T cell factor. Further, modes of binding of the fabricated inhibitors were explored using SDM (site-directed mutagenesis studies) and structure-activity relationship studies. Yu et al. thereby provided a novel approach for designing inhibitors that can effectively bind with β-catenin and disrupt its interaction with TCF (Yu et al. 2013).

6.6.3.2 Designing of Inhibitors Based on Secondary Structures Involved in PPI

Peptide and peptidomimetic-based strategies are highly effective in designing inhibitors against PPIs for which endogenous binders are not available. Handful applications of these strategies have been marked, although their clinical applications are limited owing to their poor cell permeability and low bioavailability of these peptide/peptidomimetic-based modulators (Wilson 2009). Hence, considerable attention is being earned by nonpeptidic secondary structure mimetics (Dewal and Firestine 2011). Most of the interactions among the proteins are mediated through the common secondary structure elements including α-helix, β-strand, and β-turn. Thus, designing small molecules that mimic the interactions among these key elements displays an alluring approach for the modulation of PPIs. As discussed earlier, α-helix is the most common secondary structure that is involved in interactions among proteins (Bullock et al. 2011). Numerous small-molecule α-helix mimetics have been fabricated to target the crucial PPIs including p53/MDM2 (Yin et al. 2005; Plante et al. 2009) and Bcl-xL/Bax (Yin et al. 2005; Azzarito et al. 2013; Whitby and Boger 2012). Such PPI inhibitors mimicking α-helices have been designed based on the structures of these scaffolds including trispyridylamide (Baker and Der 2013), benzamide (Itoh et al. 1999), terpyridine (Itoh et al. 1999), terphenyl (Jiang et al. 2014), and tetraphthalamide. However, such terphenyl-based mimetics are highly insoluble in water. They also require long synthetic routes and also suffer from restricted flexibility. Hence, keeping in view the limitations of terphenyl-based compounds, it is essential to design α-helix mimetics that exhibit high cell permeability, flexibility, and their preparation involves an easy and short synthetic process.

Attempts have been made to design novel α-helix mimetics which possess high solubility in aqueous medium (Hajduk and Greer 2007; Erlanson et al. 2004b; Swayze et al. 2002; Lepre et al. 2004; Siegal et al. 2007; Schuffenhauer et al. 2005; Zimmermann et al. 2014). Most of the strategies that aim at increasing the

solubility of scaffolds rely on replacing heterocycle benzene. For instance, Cummings et al. have fabricated a new scaffold known as 5-6-5 imidazole-phenyl-thiazole core exhibiting high water solubility. Inhibitor designed based on this scaffold has shown inhibitory actions against Cdc42/Dbs PPI at micromolar concentrations, and hence can be designed as an anticancer agent (Cummings et al. 2009). Further, Moison et al. have synthesized nonpeptidic scaffold using an approach that employs pyridazine core. Authors synthesized urea-pyridazine-piperazine scaffold which is highly versatile. It has been proposed that such scaffolds can be utilized to design small libraries of low-molecular weight molecules mimicking α-helices that can be used to target specific PPIs (Moisan et al. 2007). Moison et al. have also fabricated nonpeptidic α-helix mimetic based on tricyclic oxazole-pyrrole-piperazine scaffold. Scaffolds possess both hydrophobic and hydrophilic parts. The hydrophobic surface aids in recognition of the target protein, and the hydrophilic edge contributes toward the solubility of the compound. This scaffold follows a modular approach for its synthesis, and hence can be designed to target wide variety of PPIs (Moisan et al. 2008). Lao et al. have developed oxopiperazine dimers that mimic the activation domain of p53 and HIF1α to tailor ligands that can interact with MDM2 and p300/CBP respectively to block their interactions with their cognate partner proteins (Lao et al. 2014).

Fabrication of low-molecular weight secondary structure mimetics has also gained importance in the field of designing PPI modulators especially in those cases lacking structural details. Although, the current approach is having several merits, it also has certain limitations including: (1) It is essential to build libraries containing secondary structure mimetics for HTS of hits-targeting PPIs. (2) Only few secondary structure mimetics have shown therapeutic efficacy in vivo, and it requires tedious efforts to convert these mimetics into the functional leads. (3) It is highly imperative to determine the subtle difference in the binding clefts of PPIs to design inhibitors that are selective toward the particular PPI. Designing modulators against PPIs that share conserved motifs is cumbersome, as it requires extra efforts to design modulator with sufficient selectivity toward desired PPI (Davis et al. 2007). In order to address this issue, it is essential to perform in-depth SAR studies (Davis et al. 2007). For instance, Shaginian et al. reported that rationally designed α-helix mimetics showed inhibitory actions against both p53/HDM2 and Bcl-Xl/Bak (Yin et al. 2005; Shaginian et al. 2009). Barnard et al. designed a library of N-alkylated aromatic oligoamide helix mimetics which are found to be selective PPI inhibitors (Barnard et al. 2015).

Small-Molecule Library of Secondary Structure Mimetics

Whitby and Boger have taken an initiative to construct a comprehensive library containing three major secondary structure motifs including α-helix, β-turn, and β-strand that are involved in PPIs (Whitby and Boger 2012). Authors designed three libraries, each specifically dedicated for three differential secondary structure mimetics. Searching all these libraries would yield the compounds that can mimic the important interactions involved in most of the PPIs. Hence, one can easily scan these libraries to search compounds that can specifically target the desired PPIs.

α-Helix mimetic library contains around 8000 members, in contrast, β-turn mimetics library contains 42,000 members. Screening of such libraries not only provides leads against PPIs, but also allows one to understand the recognition domain and key residues involved in interaction. Screening these libraries for obtaining hits against p53/MDM2, HIV-1/gp41, and opioid receptors has resulted in the hits which mimic their endogenous ligands. Such attempts have resulted in α-helix mimetics against HIV-1gp41 and β-turn mimetics against opioid receptors. Both mimetics have shown high-binding affinity (in micromolar and nanomolar range) for their target proteins. Further, their SAR studies have not only provided information about the crucial residues of interaction; however, it has also provided a treasurable information regarding the binding mode preferences that further aids in optimizing the structure of leads. Hence, it provides an efficient method not only for obtaining small molecule leads and their validation and optimization avenues, but also facilitates the interrogation of PPIs (Whitby and Boger 2012).

Computational Designing of α-Helix Mimetics Against p53-MDM2 and p300-HIF1α

Lao et al. have developed a computational protocol to design peptidomimetic-based inhibitors (Lao et al. 2014). They have used their protocol for developing inhibitors against two important PPIs namely, p53/MDM2, and p300-HIF1α. The major aim of computational design is to scale down the total number of designs that can be aptly managed, synthesized, and tested experimentally. For instance, there are four variable positions in oxopiperazine, which can be substituted with combination of 17 amino acids (20 canonical amino acids without Pro, Gly, and Cys). Considering four variable positions, and 17 amino acids, there will be more than 83,500 possible combinations of designs. The present calculation of number of possible designs is not considering noncanonical amino acids. The inclusion of such noncanonical amino acids will further result in significant increase in number of possible designs. Thus, synthesis and experimental validation of such vast number of designs are quite cumbersome. Hence, computational approach provides a means to reduce the number of such designs to synthesize and for experimental validation.

Lao et al. took an initiative to work toward this goal by combining computation designing of proteins with peptidomimetics scaffolds for the development of OHMs (oxopiperazine helix mimetics) as inhibitors of PPIs. They have employed Rosetta in optimizing the binding affinities of oxopiperazine mimetics. Basic steps involved in designing inhibitors using Rosetta include identification of set of residues and side chain conformations exhibiting low energies when a target with fixed backbone constraints and a flexible ligand is used (Fig. 6.12). More precisely, the key steps include: (a) identify locations on the scaffold that can mimic hot spot residues; (b) experimentally validating the scaffold that contains hot spot mimics; (c) optimization of leads and design of hot spot analogs using computational algorithm; (d) experimentally validating the molecules bearing top ranks (Fig. 6.13). Several features have been added in Rosetta to ease the process of designing inhibitors. For instance, computational complexity has been reduced by representing the side chains as rotamers. Further, Rosetta framework has been

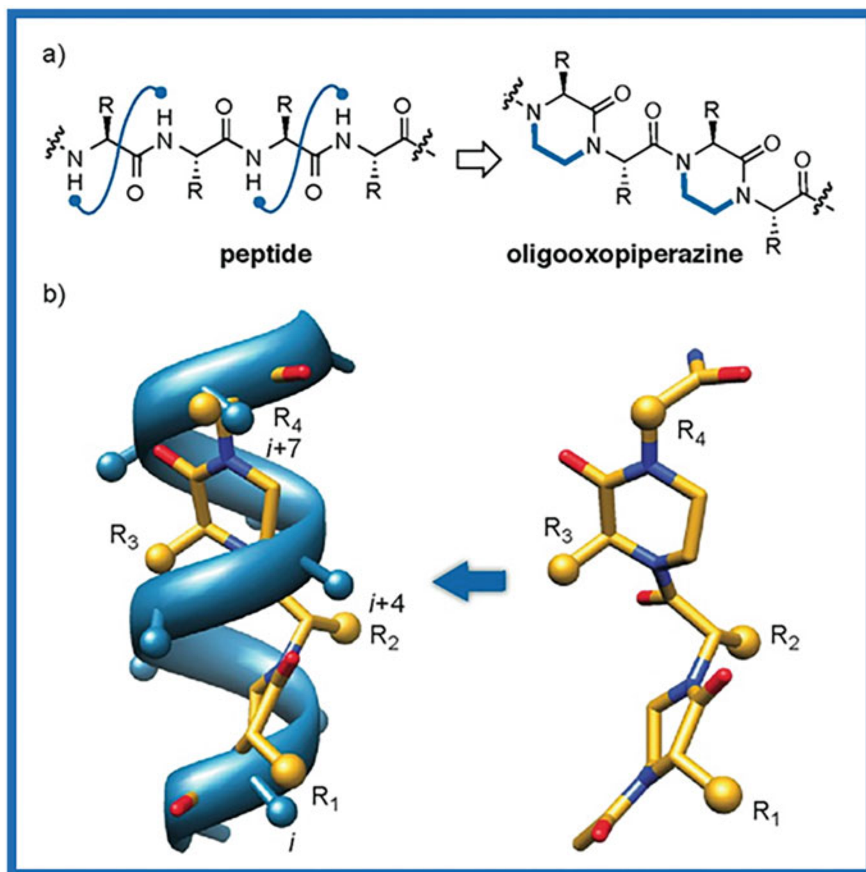

Fig. 6.12 Structural schematic depicting (**a**) the formation of a peptidomimetic oligooxopiperazine, and (**b**) an overlay of 8-mer canonical a-helix and dimeric form of oxopiperazine (left). Predicted low energy structure of dimeric oxopiperazine (right)

extended which allows user to model and design noncanonical amino acids on nonnatural scaffolds including peptoids (Lao et al. 2014; Drew et al. 2013; Butterfoss et al. 2009; Renfrew et al. 2012; Gulati and Poluri 2019; Poluri and Gulati 2017a). They have also introduced several methodologies in Rosetta suite to conformationally sample the scaffolds. For instance, conformational sampling of oxopiperazine can be easily carried out. Novel scoring function which is based on molecular mechanics has been introduced and is not based on protein centric knowledge-based score terms, which adds an additional advantage in the designing process (Renfrew et al. 2012). New editions of Rosetta also allow the researchers to model differential set of molecules in addition to nucleic acid and proteins (Drew et al. 2013). Further, a new function has been added to Rosetta that allows efficient conformational sampling of oxopiperazine including puckering of its ring structure (Drew et al. 2013).

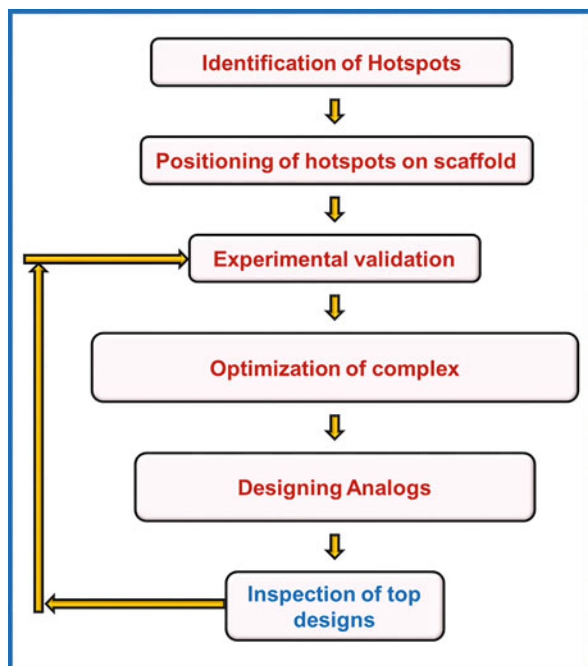

Fig. 6.13 Flowchart depicting the development of various conformations of a flexible ligand and analyzing their mode of action in PPI inhibition

Lao et al. have designed inhibitors that are highly potent against p53/MDM2 and p300-HIF 1a. In case of p53/MDM2 inhibitor designing process, three hot spot residues F19, W23, and L26 were considered to initially model them on oxopiperazine scaffold. Dimeric form of oxopiperazine displayed four amino acids. Computational modeling studies have shown that first, second, and fourth side chains which are labeled as R1, R2, and R4 respectively in Fig. 6.12 displayed good overlapping with i, $i + 4$, $i + 7$ side chains of α-helix. This indicates that such positions are in well concordance with F19, W23, and L26 (hot spot residues) of p53. R3 group which is not in direct contact with MDM2 can be utilized to locate a residue which increases the solubility profile of the mimetic. This position can be used to locate a small noninteracting residue. Locating Alanine at R3 position has resulted in a mimetic which has K_d of 65 μM. Further, computational modeling is used for investigating the mode of binding and for the prediction of molecules which exhibit high-binding affinity. Top models were selected on the basis of binding energy, mode of binding, synthesis feasibility, and other physiochemical features. Experimental validation of such top designs was carried out, and it was observed that Rosetta returned high-affinity binders. For instance, a molecule with a binding affinity of 0.3 mM for MDM2 has been obtained. This approach has also been applied to design inhibitors against p300/HIF-1α. Three hot spot residues including L818, L822, and Q824 on HIF-1α helix was used. R1, R2, and R4 positions on oxopiperazine were predicted to mimic such hot spot residues. Lead compound with these residues showed a K_d value of 533 nM for the CH1 domain p300. Further,

computational modeling suggesting the replacement of leu with norleucine has resulted in inhibitor with 13 times improvement in its binding affinity (K_d, 30.2 nM). This marks the importance and advantage of using Rosetta in designing the high-affinity and more specific inhibitors against PPIs (Lao et al. 2014). Rosetta software is freely accessible for academic researchers through Rosetta Commons website.

Rational Designing of α-Helix Mimetics Against c-Myc/Max

Novel α-helix mimetics have been designed to interfere the formation of heterodimer between c-Myc and Max. It has been proposed that inhibition of heterodimerization results in inhibition of oncogenic actions carried out by Myc. Hence, inhibitors have been designed against c-Myc/Max interaction exhibiting high therapeutic values (Hammoudeh et al. 2009). Presence of high α-helical content in the c-Myc/Max heterodimer indicates that designing synthetic α-helix mimetics can hinder the heterodimer formation (Fieber et al. 2001). Jung et al. worked in this direction and rationally designed α-helix mimetics with low-molecular weight and with an ability to recognize and interact with helical c-Myc in its transcriptionally active coil-coil structure associated with Max. Such inhibitors can obstruct the interaction of heterodimer with its canonical E-box DNA sequence. Hence, these α-helix mimetics block the function of PPI without actually causing the dissociation of proteins. Of all α-helix mimetics, few showed high selectivity toward c-Myc-Max heterodimer possessing an IC_{50} value less than 5.6 μM in contrast to homodimer of max (Jung et al. 2015).

6.7 Virtual Screening

In the current modern computing era, computational tools are being developed to assist researchers as the rapid and efficient approaches for modern drug discovery. Virtual screening also known as in silico screening has evolved as complementary technique to facilitate the high-throughput screening for the development of pharmaceuticals. Virtual screening aids in decreasing the number of compounds that need to be screened in bioassays. Hence, virtual screening efforts contribute in reducing the time and cost during drug development process (Lionta et al. 2014; Basile 2018). Virtual screening approaches have been categorized into two namely: (a) structure-based virtual screening (SBVs), and (b) ligand-based virtual screening (LBVS), on the basis of availability and usage of information about the targets. LBVS takes into account the structural information about at least one known active compound or ligands against the target protein. In contrast, SBVS considers the structural information of the target to design the inhibitor. LBVS approach includes several methods like pharmacophore-based methods, 3D shape matching, and quantitative structure-activity relationships (Villoutreix et al. 2007). In contrast, SBVS approach majorly involves molecular docking to the target structure. Both LBVS and SBVS are described in detail in the following sections. Both LBVS and SBVS approaches have their own merits and demerits, which are summarized in Table 6.2.

Table 6.2 Summary showing the merits and demerits of structure-based virtual screening (SBVS) and ligand-based virtual screening (LBVS) approaches

Approaches	Methods	Merits	Demerits
SBVS	Molecular docking	No biases toward the available ligand structures	Probably high false-positive outcomes
	Binding site comparisons	Considers flexibility of proteins	Simple scoring functions
	Pharmacophore-based models	Employs protein structure details	High screening time
LBVS	QSAR modeling	Computationally less intensive	Less accurate
	Similarity methods	Simple and fast	Requires information about present ligands
	Pharmacophore-based modeling	Structural details of proteins not requisite	No consideration of protein structural framework

Virtual screening methods have gained much attention by researchers across the globe for its utility in designing inhibitors against the target proteins. For instance, Sun et al. found 12 compounds that act as inhibitors against sirtuin (SIRT1) using virtual screening approach (Sun et al. 2016). Yang et al. employed structure-based virtual screening method to design inhibitor against KDM5A (Yang et al. 2019). Wu et al. repurposed mitoxantrone as a NAE inhibitor by virtual screening of drug database which is FDA approved (Wu et al. 2018). Virtual screening has also gained importance in screening inhibitors against PPIs. For instance, Yang et al. employed SBVS to screen inhibitor against VHL/IHF1α interaction (Yang et al. 2016). Zhong et al. found cytosine alkaloid to block the interaction between menin and MLL (Zhong et al. 2016). The same group have also designed inhibitors against heterodimer of TLR1-TLR2 using SBVS approach (Zhong et al. 2015). In a similar fashion, the approach has also been utilized to galvanize the interaction of flavonoids with histone like DNA-binding protein (HU) of Helicobacter pylori and few chemokines such as CCL2, CXCL3 followed by correlation with the NMR-based titrations and fluorescence spectroscopy (Joshi et al. 2020; Raj et al. 2020, 2021).

6.7.1 Ligand-Based Virtual Screening (LBVS)

Ligand-based virtual screening is based on the physiochemical features and structural details of scaffolds from the known active and inactive molecular entities. These features are closely monitored under the principles of molecular similarity (Johnson and Maggiora 1990). Suitable molecular descriptors are used to closely examine the level of similarities among the molecules to unravel the relationships between the compounds contained in the library, and the known active and inactive molecules. Both 1D and 2D descriptors encompassing information with respect to chemical features of compounds, and their topological characteristics (Duan et al. 2010; Ivanciuc et al. 2000; Jørgensen and Pedersen 2001) are being employed for the

similarity measurements. 3D descriptors have also been designed which contain information associated with shape, volume (Sastry et al. 2011; Hawkins et al. 2007), molecular fields (Vázquez et al. 2018; Cheeseright et al. 2008; Mestres et al. 1997; Cross et al. 2010), and pharmacophores (Cross et al. 2010; Abrahamian et al. 2003) for the further improvement in LBVS approach. LBVS strategy includes three different approaches including: (1) QSAR (quantitative structure-activity relationship) modeling; (2) similarity of compounds; and (3) pharmacophore modeling. Each of these approaches are described briefly in the following sections.

6.7.1.1 QSAR Modeling

QSAR modeling is a method to construct a computational/mathematical model. The method relies on retrieving a statistical correlation between the structural and physiochemical characteristics of ligands and their biological actions. Several QSAR approaches have evolved over a period of 100 years and developed as a valuable predictive tool specifically in the field of designing pharmaceuticals. QSAR methods with different dimensions including 1D, 2D, 3D have evolved, and each of them has their own specific applications. All the 1D, 2D, and other related QSAR models are known as classical methodologies of QSAR. Each of these QSAR formalism considers that each molecule involved in the study shares the same interaction site of the particular target protein. Each of these formalisms differs in a manner in which they treat and display the structural properties of the molecules and extract the associations between their characteristic features and activities. 1D QSAR correlates the activity of molecules with global molecular characteristics including log P, pK_a, etc. In contrast, 2D QSAR involves correlation between the activity and pattern of structure that includes 2D-pharmacophores, connectivity, indices, etc. 3D QSAR involves correlation between the activity and noncovalent interaction fields that surround the molecule (Verma et al. 2010).

QSAR represents the first ligand-based approach for screening of compounds with desirable properties. QSAR modeling relies on one major hypothesis according to which both 2D QSAR and 3D QSAR properties of ligands are employed for the construction of statistical model depicting the biological activity of ligands. This model is then employed to retrieve the biological activities of new compound. The major limitation of 2D QSAR is that they do not consider the spatial location of physiochemical properties of the ligands. 3D QSAR models are also not devoid of limitations. 3D QSAR also necessitates the information like biologically active conformation of the compounds and their alignment for the construction of model. Further, it is also important to note that 2D-based algorithms are fast; however, they are less accurate in contrast to 3D-based QSAR algorithms. However, 2D QSAR methods are not able to determine new active compounds with dissimilar structures (Stumpfe et al. 2012). Moreover, both 2D and 3D QSAR model constructions does not take into consideration several important properties including conformation of ligand, structure of proteins, their flexible nature, and solvation effects (Drwal and Griffith 2013).

6.7.1.2 Similarity Methods

Similarity methods represent another ligand-based approach for virtual screening. This method is quite simple and cost-effective for the retrieval of compounds encompassing features similar to the known ligands. These features are encrypted as 2D and 3D descriptors. For example, topological descriptors express molecular structures as fingerprints. Descriptors containing information about the molecular shapes have also been designed. Both 2D and 3D methods have shown successful applications in virtual screening. Such similarity methods have shown to outperform the docking methodologies, while taking into consideration of the enrichment and computation time for differential targets (Hu et al. 2012). However, such 2D and 3D similarity methods are highly influential by users. This is due to the reason that it is difficult to choose the input molecules. Once the input molecules have been selected, the method will be biased toward the selected molecules. Hence it is essential to carefully choose the input structures to obtain the better outcomes. Choice of input molecules is mostly carried out by employing the common chemical characteristics from 3D structures of known ligands that display their interactions with target (Drwal and Griffith 2013). Choice of descriptors also highly influences the screening performance. 3D descriptors, specifically, shape descriptors can also be employed for scaffold hopping, that involves changing the scaffold while retaining its activity (Reid et al. 2008; Rush et al. 2005; Sheridan and Kearsley 2002; Bajorath 2001; Montes 2009).

6.7.1.3 Pharmacophore-Based Modeling

Pharmacophore modeling is a ligand-based approach which is based on pharmacophores designed on the basis of chemical structure of ligands. Pharmacophores basically represent the spatial organization of various chemical features including the hydrogen bond acceptors and donors of the ligands that are essentially required for binding its target protein (Leach et al. 2010). Ligand-based pharmacophores represent 3D QSAR models that are highly imperative in those cases where there exists limited structural information with respect to target proteins, and also there is a lack of details about the active conformation of ligands. Pharmacophore model is constructed using the set of ligands that are structurally and functionally diverse. Ligand-based pharmacophores are incredibly important and are also being implemented in numerous commercial molecular modeling packages including MOE (chemical computing group), Discovery studio (Accelrys), Phase (Schrodinger), Sybyl (Tripos). The main advantage associated with pharmacophore is that they can be employed for activity profiling and antitarget modeling, in addition to their usage in identification of new active compounds in virtual screening (Schuster 2010).

Pharmacophores refer to simple display of features essential for binding and scoring of compounds that is carried out on the basis of feature mapping and geometric fits. Strength of interaction can also be considered via feature weights adjustments. However, such an attempt can cause biasness and overfitting of the pharmacophore toward the input structures. Pharmacophore modeling efficiency is entirely based on the presence of good training sets that contain compounds

exhibiting the same mode of binding. Mostly, it is quite challenging to identify pools of structurally and functionally diverse set of molecules along with the availability of their quantitative activity data including their binding efficacies and IC_{50} values. It is also essential to note that the absence of publications reporting negative results also create a hurdle in identifying inactive molecules. Hence, in most of the cases, there is a qualitative common feature pharmacophores development on the basis of active molecules (Drwal and Griffith 2013). Several different software dedicated toward modeling pharmacophores have also been designed including catalyst, unity, GASP, Disco, which are being widely utilized in drug designing processes (Patel et al. 2002).

6.7.2 Structure-Based Virtual Screening (SBVS)

Structure-based virtual screening is also known as target-based virtual screening, as this method exploits the 3D structural details of the target. This method does not require the information with respect to the biological activity of the known molecule. Structure-based virtual screening includes three approaches namely: (1) Molecular docking approach; (2) Binding site comparison approach; and (3) Pharmacophoric approach. Each of these approaches is discussed in detail in the following sections.

6.7.2.1 Molecular Docking

Molecular docking is one of the most popular and widely applicable SBVS approach. This technique is applied to determine the compounds having high-binding affinity to the active sites of the target (Lexa and Carlson 2012; Andricopulo et al. 2008). This SBVS approach initially requires three-dimensional structure of a target protein and set of ligands filtered obtained from virtual screening. This step is followed by the docking of these ligands onto the target protein and scoring of these complexes to filter out the potent lead compounds such as gallic acid, glycyrrhetinic acid, epigallocatechin (Raj et al. 2020, 2021). Docking and scoring yield the compounds having higher binding affinity to the target protein (Salmaso and Moro 2018; Ghosh et al. 2006). The major advantage of this method is that it utilizes the structure of the target protein, and hence it is not biased toward the structure of ligand. Further, there is a provision to include protein flexibility measure in the docking algorithms via soft docking methods, an inclusion of side chain rotamer libraries, ensemble or induced fit docking methodologies (Meng et al. 2011; Ivetac and McCammon 2011). However, the incorporation of flexibility of proteins during docking procedure takes significantly longer time and also results in increased rate of false-positive outcomes. This is due to the reason that flexibility of target protein enables a greater number of ligands to dock into the pocket. It is important to mark that scoring of protein ligand complexes represents the major challenge of this approach. Since the scoring functions are required to compromise between the simplicity and complexity on the one side, and they have to calculate the binding-free energies with high accuracy on the other side for the highly efficient calculations. Almost, in case of all the scoring functions, the results are mostly

dependent on the target, and possess little correlation with the binding affinity of ligand. Moreover, there is an ignorance of entropic and solvation contributions toward the binding of ligands. Further, docking model attained using SBVS can also be used for analyzing interactions which can be further utilized to improve the selectivity and activity features of compounds.

6.7.2.2 Binding Site Comparisons

This is a novel approach which is used for repurposing the drugs and for polypharmacology. This method is based on the fact that differential proteins possess similar binding sites (Konc 2019). Hence, the binding sites can be scanned in any particular protein and matched with specific chemical structure.

6.7.2.3 Pharmacophore-Based Models

Pharmacophore-based approaches can also be used with SBVS. Structure-based pharmacophore can be designed on the basis of structural characteristics of binding site if the high-resolution three-dimensional structure of the target is present. In this scenario, there is no requirement of library of known inhibitors against the target. It is highly probable to identify novel scaffolds using SBVS, as it relies on physical interactions that are calculated in silico, instead of relying on similarity and dissimilarity measures of known ligands. Hence, SBVS is able to determine inhibitors that exhibit unique mechanisms of action (Leung et al. 2019; Yasuo and Sekijima 2019). Structure-based pharmacophores share merits over ligand-based pharmacophores. Moreover, such structure-based pharmacophores can be employed in the absence of ligand information, or if limited information is available with respect to ligand. Additionally, structure-based pharmacophores can be exploited to gain understanding of entire interactions in which the specific protein pocket is involved. In contrast, structure-based pharmacophores also face a difficulty as there are a greater number of interaction sites in comparison to that observed normally in protein-ligand complexes. Hence, it is important to select essential structure-based pharmacophore characteristics. Further, it is important to take into account differential protein conformations, considering which will further complicate the selection feature process and also culpable for increment in computational cost.

6.7.3 Integrated SBVS and LBVS

Integrating SBVS and LBVS techniques is a budding approach for the discovery of PPI inhibitors. Combined strategy is employed in those cases where structural data with respect to ligand-target complex as well as the similarity relationships with known active compounds are available (Sperandio et al. 2008; Talevi et al. 2009). Several research groups across the globe have used the potential of this combined strategy for designing novel inhibitors against PPIs. For instance, the authors have discovered inhibitors against HDAC8, which represents a potential drug target to treat T-cell lymphoma and neuroblastoma. They screened structurally diverse, nonhydroxamate inhibitors using the combination of pharmacophore modeling,

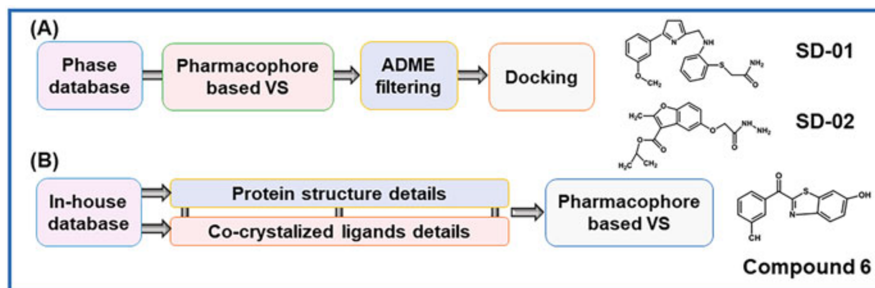

Fig. 6.14 Schematic representation depicting the identification of PPI inhibitor molecules such as (**a**) SD-01, SD-02, and (**b**) compound 6 with the help of integrated SBVS and LBVS approach

3D QSAR modeling, in-silico ADMET, and XP glide docking studies. Authors found two compounds namely SD-01, and SD-02 to inhibit HDAC8 with IC_{50} values in nanomolar range (Fig. 6.14a) (Debnath et al. 2019). Spadaro et al. designed inhibitors against 17β-HSD1, a drug target to treat estrogen-dependent diseases including breast cancer. Authors designed a pharmacophore model using the crystallographic data and exploited the model to virtually scan the small library of compounds. A moderately active compound 5 was attained after the experimental verification of hits obtained by virtual screening. This compound 5 was rigidified and structurally modified and a novel inhibitor containing benzothiazole-scaffold which is linked to phenyl ring via amide or keto bridge was obtained. Both amide- and keto-derivative compounds showed inhibition in nanomolar range against 17β-HSD1 (Fig. 6.14b). Both these benzothiazole-based inhibitor compounds have shown high potencies for their development as therapeutics (Spadaro et al. 2012). Combined strategies followed by these studies are depicted in Fig. 6.14.

Several approaches aiming at combining LBVS and SBVS strategies have been proposed to design a fortified approach. Such an approach includes the complementary features from LBVS and SBVS, and aids in resolving the discrepancies associated with each individual strategy (Wang et al. 2020; Drwal and Griffith 2013; Wilson and Lill 2011). Major limitation of LBVS is its biasness toward the template used as reference, which sometimes leads to overfitting of structures used as input. Pharmacophore model used for screening the libraries, relies on the chemical characteristics of training datasets that are used for designing an optimal pharmacophore. Moreover, presence of only limited activity data is insufficient to select structural and functional libraries of compounds. Further, molecular docking methods have a limitation related to taking into consideration the flexibility of proteins, as there exists a flexibility in the binding site of proteins due to which it can attain different conformational states. Proteins upon binding to their ligands, undergo structural changes in their loop regions and also remodel their secondary structure elements (Chen 2015; Salmaso and Moro 2018; Lexa and Carlson 2012; Spyrakis et al. 2011). Additionally, the water molecules in some cases also participate in mediating interaction of ligands to the target proteins, such water molecules

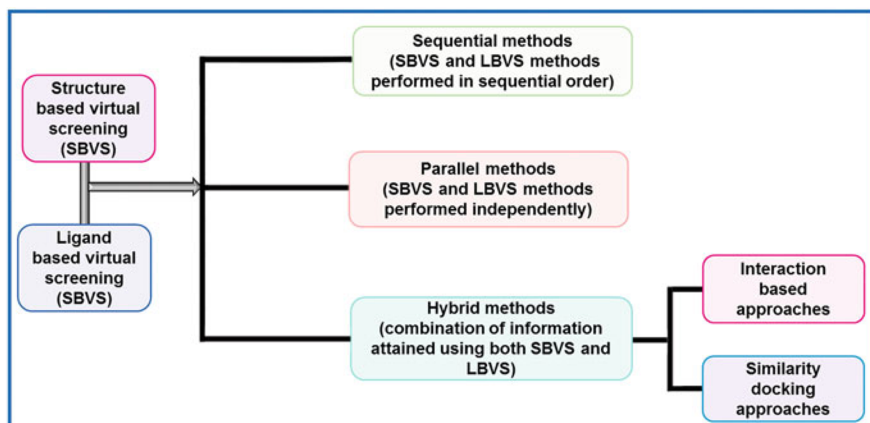

Fig. 6.15 Schematic illustrating all the three different ways in which the combinatorial approach is achieved for identification of novel PPI inhibitors

are known as bridging waters or ordered waters. These water molecules should also be taken into consideration during docking studies (Geschwindner and Ulander 2019; Maurer and Oostenbrink 2019; Schiebel et al. 2018; Rudling et al. 2018). Furthermore, docking methods also face a challenge while estimating the accurate scores and binding affinities of the complexes in a cost-effective manner (Palacio-Rodríguez et al. 2019; Guedes et al. 2018; Liu and Wang 2015; Ferreira et al. 2015). Finally, both SBVS and LBVS methods strongly depend on the target proteins (Eckert and Bajorath 2007; Hein et al. 2010).

Keeping in view the limitations of these methods, a combinatorial approach of SBVS and LBVS has been devised so that the merits of each of these methods converged and their demerits can be rectified to increase the quantity and quality of hits, while screening large chemical libraries. It has been proposed that the combinatorial approach considers all of the information available about a particular system (Hein et al. 2010; Wilson and Lill 2011). LBVS and SBVS strategies are combined in three differential ways namely: (1) sequential; (2) parallel; and (3) hybrid manner (Fig. 6.15). All these combinatorial approaches are possible with the currently available modeling software packages. Each of this approach is discussed briefly in the following sections.

6.7.3.1 Sequential Combination of SBVS and LBVS

In a sequential approach, SBVS and LBVS methods are combined in a sequential manner. This approach involves filtering of hits in a sequential manner until the most promising candidates are obtained for experimental testing. Mostly, the methods that are computationally inexpensive are used first, followed by the more expensive methods in order to reduce the overall cost. For instance, pharmacophore-based screening is carried out first in a multistep screening process. This implies that prefiltering is done on the basis of pharmacophore model. Once the number of hits

are decreased, more expensive and demanding computational approaches including molecular docking are employed to further filter out the attained hits. Numerous studies have used this strategy in which hits obtained using one or more pharmacophore screening process were further filtered using other filters including ADMET and drug likeness (Smith et al. 2012; Drwal et al. 2011; Banoglu et al. 2012; Weidlich et al. 2010; Khan et al. 2010). Finally, the hits were evaluated by exploiting docking approach. In summary, sequential approach uses LBVS for prefiltering at the initial stage and exploits SBVS methods to obtain promising hits at the final stage in a multistep filtering process. However, this type of combinatorial approach of VS is not exploiting all the available information at once and is not allowing the counteraction of limitations of each individual process.

Sequential approach has been applied by various research groups in designing inhibitors/modulators against various targets. For instance, Khan et al. employed sequential approach for the identification of GPER-1 (G-protein-coupled estrogen receptor-1) modulators. Validation of both LBVS and SBVS was carried out using Receiver Operating Curve (ROC) plot, and the early enrichment factor (EEF). LBVS was accomplished by using an agonist of GPER-1 named G1. G1 was used as a query model in order to screen eMolecules library by employing two approaches namely EON (electrostatic potential screening) (Vázquez et al. 2020) and ROCS (Rapid Overlay of Chemical Structure) (Hawkins et al. 2007). Hits with high scores obtained from LBVS were subjected for further screening via SBVS. SBVS involved structure generation for GPER-1 using homology modeling, followed by molecular docking studies of hits against the modeled structure of GPER-1. Authors obtained final hits that exhibit high scores in contrast to G1, and the scores were calculated using Chemguass 4 score. Final hits were synthesized, and their antiproliferative activities have been evaluated.

Dawood et al. have also employed sequential approach, in which the SBVS was employed initially, which was followed by LBVS (Dawood et al. 2018). Authors screened a database containing 1720 phytochemicals for the identification of inhibitors against aromatase enzyme. This initial screening was carried out using Glide docking with its "extra precision" feature (Friesner et al. 2004, 2006). Hits obtained were subjected for LBVS via a fabrication of pharmacophore and QSAR models. In vitro testing of top scoring hit revealed its aromatase inhibitory activity with an IC_{50} value of 2.2 μg/mL.

6.7.3.2 Parallel Combination of SBVS and LBVS

In the parallel approach, both SBVS and LBVS methods are performed independently. High-score hits obtained from each method are chosen for biological testing studies. Methods of SBVS and LBVS are selected in a way that each of them is complementary to each other. For instance, methods including pharmacophore modeling, similarity methods, and docking can be used in parallel combination. Svensson et al. have reported that the fusion of virtual screening methods performs better in comparison if these methods are used independently. It has also been marked that the parallel combination of SBVS and LBVS yields better results in comparison to other combinatorial approaches (Svensson et al. 2012). Swann et al.

have presented the application of this approach by designing a unified probabilistic framework for SBVS and LBVS (Swann et al. 2011). They have applied and validated this approach on more than 30 target proteins.

Tann et al. assessed the performance of parallel combination of SBVS and LBVS. They combined the similarity searching and docking methods in a parallel fashion using 2D fingerprints. They employed this approach to identify inhibitors against nine target enzymes. Combination of results from similarity searching and docking was carried out using fusion ranking and parallel selection method. In fusion ranking, ranks from both similarity search and docking were added to obtain final ranking. In contrast, in parallel selection of compounds, an alternative selection of compounds was carried out depending upon their ranks attained from similarity and docking methods independently. It was erected that parallel selection of compounds is superior in contrast to the rank fusion method (Tan et al. 2008).

Vucicevic et al. have employed parallel SB and LB screening approach to identify the compounds with an anticancer potency. Authors screened a large library containing $9*10^6$ compounds and selected high-scoring compounds from both the SBVS and LBVS methods. Selected compounds were further subjected to biological evaluation. They successfully retrieved a compound showing cytotoxicity profile similar to that of rilmenidine, and displayed significantly enhanced apoptotic activity response to doxorubicin (Vucicevic et al. 2016).

Costa et al. have recently used the parallel combination of SB and LB virtual screening in order to screen novel inhibitors against HIV-1 reverse transcriptase RNA-dependent DNA polymerase activity. Authors scanned ZINC database containing more than 143,000 natural compounds. They selected 20 hit molecules and evaluated them using biochemical assays. The authors obtained three hits as new nonnucleoside RT inhibitors with affinities lying in low micromolar range (Costa et al. 2019).

It is important to note that in contrast to sequential combination, parallel combination of SBVS and LBVS is an expensive approach, as it requires differential virtual screening techniques to be run at the same time. This is essential in order to retrieve the ranking of compounds from different methods at one go, which further aids in making the final selection of compounds.

6.7.4 Hybrid Combination of SBVS and LBVS

Hybrid methods combining SBVS and LBVS, utilizing information from both the screening approaches have been divided into two categories namely, (1) interaction-based methods; and (2) similarity-docking methods.

6.7.4.1 Interaction-Based Hybrid Methods

Interaction-based hybrid methods are based on first determining the differential protein–ligand interactions. Such interactions are further used to screen the libraries of chemical compounds by utilizing pseudo-receptor and pseudo-query methods (Tanrikulu and Schneider 2008). Pseudo-receptor methods are based on mapping all

the important interactions in which a particular set of ligands may be involved in, when they are present in their bioactive conformations. This bioactive conformation is the one that simulates their orientation in the binding pocket (Pei et al. 2005; Andrews et al. 1989; Vedani et al. 1993). This process aids in defining the structural shape and key anchoring residues in the binding pocket, which further assists in screening large chemical libraries. Hence, the chemical nature of ligand dataset as well as the superposition of ligands plays a crucial role in the performance of these models.

In contrast, pseudo-query methods extract the interaction patterns by utilizing the structural information about protein-ligand complex that is attained by experimental efforts. The extracted interaction pattern is converted into fingerprints to depict ligand–target interactions. Alternatively, such interaction pattern can also be used to design pharmacophore. Fingerprints/pharmacophores are then exploited in similarity searches to the identify ligands that follow same pattern of interactions (Koes and Camacho 2012b; Sato et al. 2010; Salam et al. 2009; Jacob and Vert 2008; Baroni et al. 2007; Wolber and Langer 2005; Pei et al. 2005; Andrews et al. 1989). Additionally, novel hits can also be identified by implying constraints with respect to volume and shape of the target-binding site. There are several pseudo-query methods that have been divided into two categories on the basis of their underlying model namely: (1) interaction fingerprint model-based methods; and (2) pharmacophore model-based selection methods. All these methods are summarized in Table 6.3.

Salentin et al. developed a computational approach that utilizes the pattern of interactions between the compounds for drug repurposing. They used PLIP methodology to analyze more than 170,000 structures of protein-ligand complexes. Authors searched a pharmacophore based on protein–ligand interaction patterns, and found an FDA-approved malarial drug (amodiaquine) as top scoring hit. The drug was evaluated using biological testing. Antimalarial drug showed inhibitory action against target protein Hsp27 and was validated as a potential anticancer agent. This implies that SBVS approach combined with PLIP interaction pattern is a promising approach for the drug repositioning (Salentin et al. 2017).

Further efforts have been made in including entropy and solvation effects. This is exemplified by the work of Tra-Nguyen et al., who have included a desolvation component of protein–ligand interaction energy in their pseudo-query pharmacophoric tool (Tran-Nguyen et al. 2019). They divided their analysis in three different steps including: firstly, druggable cavities on the surface of target protein were scanned followed by the determination of pharmacophoric features; secondly, cavity-based pharmacophore queries were generated in three-dimensional space; and lastly, cavity-based features were exploited to generate molecular alignments using Shaper. The proposed strategy was observed to be as efficient as other virtual screening methodologies including docking especially in context of both predictions of poses and power of ranking (Tran-Nguyen et al. 2019).

Attempts have also been made in the direction of exploiting interaction fingerprints (IFP) which encompass information about the interactions of target protein with ligands. This model leads to ample declination in the computational cost of virtual screening by compressing three-dimensional structural binding details

Table 6.3 Summary of some interaction fingerprint-based and pharmacophore-based methods involved in assessment of protein–ligand interactions

Models	Methods	Summary	Reference
Interaction fingerprint-based	FLIP	Facilitates fast means of generating usable fingerprints encoding protein–ligand interactions. (http://zistrayan.com/development/download/flip/package.zip)	Hajiebrahimi et al. (2017)
	PLIP	Facilitates the automate detection and visualization of seven differential types of protein–ligand interactions in three-dimensional structures. (projects.biotec.tu-dresden.de/plip-web) (protein–ligand interaction profiler)	Salentin et al. (2017)
	PADIF	Novel approach designed to utilize GOLD scoring function in combination with other specific scoring schemes (protein per atom score contributions-derived interaction fingerprint)	Jasper et al. (2018)
	SIFt	Analyzes binding interactions between protein–ligand, converts interaction fingerprint into binary strings, effective tool to filter molecules with specific binding modes/interaction patterns with protein target. (structural interaction fingerprint)	Deng et al. (2004)
Pharmacophore-based	FLAP	Analyzes the four factors of pharmacophore fingerprint in addition to their shape component for the understanding the protein–ligand interaction (fingerprints for ligands and proteins)	Cross et al. (2010)
	IChem	Analyzes the protein–ligand interaction and creates their fingerprint and graphs	Da Silva et al. (2018)
	LigandScout	Analyzes the six different nonbonded interactions taking place between protein and ligand and volume constraints	Wolber and Langer (2005)
	TIFP	Analyzes the protein–ligand interaction to provide a string of triplets consisting of two interacting and one interaction pseudoatom	Desaphy et al. (2013)

into a one-dimension binary string. Deng et al. presented a novel method SIFt (structural interaction fingerprint) to represent and analyze the protein–ligand interaction data. Authors have shown that SIFt methodology that exploits interaction fingerprint patterns can be used as an effective filter tool to virtually screen large chemical libraries, and to choose molecules with desirable modes of binding or to select the molecules that are involved in desirable pattern of interactions with target protein. Hence, SIFt can be employed as an effective tool in virtual screening of inhibitors against specific PPIs (Deng et al. 2004).

6.7.4.2 Similarity Docking-Based Hybrid Methods

It is a highly challenging task to assign an appropriate score and rank the compounds during virtual screening. To address this issue, several researchers have amalgamated similarity search measurements and molecular docking for accurately assigning the scores and ranks to the sampled ligand poses (Kelley et al. 2015; Lee et al. 2008; Kumar and Zhang 2016a, b, c, 2019a; Prathipati and Mizuguchi 2016). Several tools including Hybrid (McGann 2012) and HomDock (Marialke et al. 2008) have been designed by exploiting the synergistic efforts of molecular similarity and docking approaches.

Predicting the Pose of Ligands

One of the interesting and straightforward application of the hybridized molecular docking and similarity search is advancement in the determining the pose of ligand in the protein-binding pocket. Considerable improvement in prediction of binding poses has been observed especially in those cases in which experimental details of modes of binding of active compounds in the target-binding pocket are available (Gathiaka et al. 2016; Gaieb et al. 2018, 2019). Kumar and Zhang developed two methods including CDVS (Cross-docking-based Virtual Screening) (Kumar and Zhang 2018) and PoPSS (Pose Prediction using Shape Similarity) (Kumar and Zhang 2016b) to exploit three-dimensional shape similarity of ligands for the prediction of ligand pose and virtual screening. CDVS employs the shape similarity to choose a receptor structure that is suitable for docking. In contrast, PoPSS selects a ligand conformation which is highly similar in shape as crystal ligand. This method places the selected ligand into the binding pocket of target protein. The complex is further refined by side chain packing and energy minimization using Monet Carlo module. The major drawback of this method is associated with the generation of ligand conformation and the scoring scheme employed. Moreover, PoPSS does not allow sampling of ligand conformation once it is placed in the target-binding pocket. PoPSS majorly depends on conformation generation methods for the attainment of native-like conformations. To address these issues, a modified version of PoPSS was developed, which is known as PoPSS-Lite. In this version, there was a replacement of side chain repacking with a simplified grid-based minimization of energy. PoPSS-Lite also allows the terminal functional group sampling while retaining the overall scaffold structure. An improvement in shape similarity conformations was also observed by the utilization of PoPSS-lite since it increases the number of ligand conformations and exploits a differential similarity metric. It has been marked that PoPSS-lite is superior in contrast to CDVS and PoPSS as it showed lower average root mean square deviation (Kumar and Zhang 2019b). Kumar and Zhang also tried to further improve POPSS and PoPSS-Lite methods by developing their upgraded version known as PoPSS-PB module. This method considers the water molecules that play an essential role in protein–ligand interactions by the incorporation of Poisson-Boltzmann solvation model. Considerable improvement has been observed in prediction of poses by using PoPSS-PB as compared with PoPSS and PoPSS-Lite (Kumar and Zhang 2019a). It has been suggested that it is essential to ameliorate the

performance of the conformation generation methods to further improve shape similarity-guided pose predictions.

Similarity Guide Scoring Scheme

Similarity measurements are also being employed to improve the ranking of the docked compounds. It has been shown that ligand-based 3D shape matching programs perform better than docking approaches. Attempts are being made to supplement the scoring functions in docking calculations with 3D molecular similarity measurements to determine the final ranks in the process of virtual screening. This effort will improve the ranking of the screened ligands. Such a combination of ranking by similarity measurements and docking was first implemented by Marialke et al. by introducing a highly efficient method known as GMA (Graph-based Molecular Alignment). Authors developed a program known as HomDock that combines GMA with optimization methods in docking. This program utilizes GMA in order to place a ligand on a rigid template, followed by the optimizing of its position in the protein field and ranking the ligands in accordance to their interactions with target protein and structural similarity to the known ligand (Marialke et al. 2007, 2008).

McGann et al. introduced a program known as HYBRID which is an exhaustive search algorithm that treats both the ligand and target proteins as rigid. HYBRID takes into account the protein flexibility by considering the multiple conformers of ligand and target proteins. HYBRID utilizes a ligand-based scoring function known as the CGO (chemical Gaussian Overlay). Scoring by CGO depends on degrees of match between the docked molecule and crystallographic ligand bound to the active site in terms of their shape, and three-dimensional arrangement of chemical characteristics (McGann 2011, 2012). Anighoro and Bajorath have also marked that combining the 3D similarity methods including 3D shape similarity of ligands and ligand–protein interaction patterns with docking procedures aids in improving the scores calculated using conventional scoring force fields during docking process. Such a combination yielded better active compounds against the target proteins (Anighoro and Bajorath 2016).

6.8 Challenges in PPI-Based Drug Discovery

Efforts have been made in the era of designing selective inhibitors against PPIs. For instance, Ji along with his companions developed two high-throughput functional assays for the quantification of inhibitor selectivity between the complexes of proteins including β-catenin/cadherin, β-catenin/Tcf, and β-catenin/APC (Zhang et al. 2013a). Ji and his group have also devised two computational methods known as sitemap (Halgren 2009) and MCSS (Multiple copy simultaneous search) (Zoete et al. 2005) that can identify the selective-binding site to target β-catenin/Tcf. They employed these methods and found small-molecule inhibitors against β-catenin/Tcf. This inhibitor exhibits dual selectivity for β-catenin/Tcf over β-catenin/cadherin and β-catenin/APC interactions (Huang et al. 2014).

Although immense progress has been made in the era designing inhibitors against PPIs, there are several challenges that are required to be addressed. ***Firstly***, the interfacial region of PPIs that plays a crucial role in drug designing process is flexible. There is a requirement of relatively exposed inhibitor due to flat PPI surface. The Ligand efficiency of PPI inhibitors is lower than the inhibitors designed for conventional targets (Gowthaman et al. 2013; Carlson et al. 2008). When inhibitors bind to the target, conformational changes occur in the target protein surface which thereby results in formation of differential pockets, in which the inhibitors can also bind in a buried form. However, such a flexibility phenomenon is not known about the protein, hence it poses a problem for structure-based designing of inhibitors or virtual screening of inhibitors. In such cases, there is need to improve ligand efficiency of inhibitors. It is also essential to generate ensembles of low-energy conformations and determine a suitable pocket where the inhibitor can bind in order to design inhibitors against PPIs. Highly selective PPI inhibitors can be designed by taking into account of the distinctive pockets that are conserved in a particular family of proteins. Several computational tools like Rosetta (Leaver-Fay et al. 2011), have been designed to efficiently detect such pockets on the protein surface that can used for designing drugs (Leman et al. 2020; Kozakov et al. 2011).

Secondly, attempts have to be made in devising new software and techniques suitable to search inhibitors for weakly interacting PPIs covering large contact areas. It is a highly daunting task to fabricate robust HTS assay for the detection of binders. Novel methodologies including those based on fragment screening, high-content screening (HCS), and high-throughput screening of multiprotein complexes have been introduced to increase the number of druggable PPIs (Miyata et al. 2010; Cesa et al. 2013). ***Thirdly***, selectively targeting PPIs by small molecules is also major challenge. In PPI networks, specific interface can interact with multiple proteins and hence inhibitors designed for the particular interface will not only block the specific PPIs, but also affect other PPIs involving that particular interfacial region. To address this problem, it is essential to establish novel assays for quantifying selectivity of inhibitors against differential protein-protein complexes and decoding selective binding sites.

6.9 Conclusions

PPIs, playing imperative role in diverse cellular process, serve as promising therapeutic targets for the treatment of several diseases. Unraveling the structural biology of PPIs has greatly geared up the exciting field of drug designing for targeting PPIs. Further, noteworthy progress has been made in the era of developing the novel HTS assays, complex chemical libraries, and computational tools to assist in designing the modulators of PPIs. Additionally, tremendous efforts have also been made to assess the specific-binding pockets on the proteins for designing selective modulators against PPIs. In short, PPI-based discovery of drugs is continuously evolving from its infancy to a maturity phase. Enrichment in the knowledge about PPIs and their

modulators will pay an astonishing future for pharmaceutical companies to access highly challenging and powerful PPI targets.

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7 Small-Molecule Inhibitors of Protein–Protein Interactions as Therapeutics

7.1 Introduction

PPIs are vital for the majority of biological processes. Human interactome involves around 130,000–650,000 types of PPIs, which implies that they represent an affluent source of drug targets (Lu et al. 2020a; Ryan and Matthews 2005). Hence, tremendous efforts are being made in the direction of designing drugs against PPIs to treat diseases (Ryan and Matthews 2005; Díaz-Eufracio et al. 2018; Ivanov et al. 2013; Shin et al. 2020). As discussed in the previous chapter, designing drugs which target specific PPIs is quite precarious in contrast to those designed against conventional targets. This is due to the fact that PPI interface is flat, and large which is mostly composed of hydrophobic and charged residues (Stites 1997; Lawrence and Colman 1993). All these features make it cumbersome for the inhibitors/modulators to block the interactions between the partner proteins. An effective inhibitor designed against PPI should be able to mask large surface area and interact with hydrophobic residues at the PPI interface (Modell et al. 2016). However, such a modulator may have large size and poor solubility due to which they have to confront with pharmacokinetic issues. It is also a highly daunting task to search for a good initial point to fabricate PPI modulators/inhibitor, since, natural ligands/binders are not available for PPIs (Smith and Gestwicki 2012). Apart from this, PPI interfaces involve noncontagious amino acids, thus the protein itself cannot be used effectively as a template to design a drug (Scott et al. 2016; Wells and McClendon 2007). Further, designed modulator/inhibitor targeting a PPI requires extensive biological experiments for their validation as a drug (Ferrari et al. 2013; Berg 2003; Lu et al. 2020a).

Despite the abovementioned challenges, tremendous advancements have been made in revelation, and characterization of PPI modulators/inhibitors. As discussed in the previous Chap. 6, differential approaches are being followed for the discovery, designing, and validation of PPI inhibitors. This is due to the fact that researchers have geared up in understanding the mechanism of actions of PPIs, and subsequently in development of PPI modulators (Lee et al. 2019; Kahan et al. 2021; Lawson et al.

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K. M. Poluri et al., *Protein-Protein Interactions*,
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2021; Nevola and Giralt 2015). They have unraveled the structural and functional details of hot regions involved in interaction of the protein partners. These hot regions are also known as hot spot residues, representing those key residues that play a critical role during the interaction of proteins. Discovery of such hot spots has reduced the surface area required to be covered by the inhibitor. Such an attempt has accoutered the researchers to design inhibitors that are specific to the hot spots to modulate the PPI (Moreira et al. 2007; Geppert et al. 2011). Further, observation of dynamic flexibility for PPI interface has also added a glare in the process of designing PPI inhibitors (Jubb et al. 2015).

Sterling efforts have also been made in the era of screening approaches including high-throughput screening, virtual screening, fragment-based screening which have further accelerated the process of designing PPI inhibitors (Vázquez et al. 2020), thus resulting in identification of significant number of inhibitors (Choi and Choi 2017). Amongst the numerous PPI inhibitors designed, some have already been approved by FDA, and some of them are at different stages of clinical trials (Petta et al. 2016; Davenport et al. 2020). Successful stories pertaining to design the variety of inhibitors targeting specific PPIs including p53/MDM2, Hsp90, β-catenin/TCF4, c-Myc/Max, KRAS/PDE δ, CD40/CD40L, Skp2/Skp1, Keap/Nrf2, PD-1/PDL-1, PPIs related to 14-3-3, and GTPases that possess high prospect for future drug discovery and development are also highlighted in the following sections.

7.2 Targeting p53/MDM2 Interaction

p53 is a tumor suppressor protein which participates in regulating numerous cellular functions such as cell cycle, DNA repair, apoptosis, and senescence (Vazquez et al. 2008; Chène 2003; Vousden and Lane 2007; Vogelstein et al. 2000). p53 gene was first identified in 1979, and it was observed that the gene is nonfunctional or misfunctional in a variety of human cancers. This happens due to the following reasons: (1) mutation in p53 is the culprit for the loss of function/improper functioning, and (2) overexpression of *mdm2* gene (Momand et al. 1998). MDM2 is a murine double minute 2 protein that was discovered in 1987, and is known to interact with p53 to block its function as transcription factor (Momand et al. 1992). There is an autoregulatory feedback loop function between MDM2 and p53, MDM2 interacts with transcriptional activation domain of p53 and blocks its activity to control the expression of other genes, and thereby p53 exerts its antiproliferative effects. In contrast, p53 binds to *mdm2* gene to regulate its expression.

The structure of p53/MDM2 complex has been solved using X-ray crystallography. It was speculated that MDM2 contains a hydrophobic cleft which is accessible to p53 for its interaction and binding in the form of amphipathic α-helix. p53-containing hydrophobic residues including F19, L22, W23, and L26 are involved in interactions with hydrophobic pocket in MDM2. Apart from hydrophobic interactions, p53 and MDM2 are also involved in hydrogen bonding interactions. Backbone amide of F19 of p53 is indulged in hydrogen bonding with Q27 residue of MDM2 at the entry of the cleft. Indole NH of W23 of p53 is involved in interaction

with backbone carbonyl of L54 of MDM2 via hydrogen bonding. L54 is located deep inside the hydrophobic cleft. Major interactions between p53 and MDM2 are mediated by L26, W23, and F19 of p53. Specific interactions pertaining to the formation of complex between p53 and MDM2 have been explored extensively. It has been observed that the amino-terminal region of transactivation domain of p53 is engaged in interactions with N-terminal domain of MDM2 (Chen et al. 1993). It has also been shown that the domain of MDM2 that is involved in binding with p53 spans residues from 1 to 52 and domain of p53 that binds with MDM2 spans residues from 1 to 118.

Several different approaches have been employed to design variety of inhibitors to regulate the interactions between p53 and MDM2 (Zhang et al. 2021; Kuusk et al. 2020; Liu et al. 2019). Some of these inhibitors are discussed briefly in the following subsections.

7.2.1 Natural Products as Inhibitors of p53/MDM2

Duncan et al. have isolated a novel peptide known as chlorofusin via the fermentation broth of *Microdochium caespitosum* during an activity-guided screening program that involved assessment of 53,000 microbial extracts (Duncan et al. 2001). It showed inhibition of interaction between p53 and MDM2 with an IC_{50} value lying in micromolar range. It has been marked that chlorofusin interacts with N-terminal domain of MDM2 (Duncan et al. 2003). Structurally, chlorofusin I is composed of azaphilone-derived chromophore linked via ornithine's terminal NH_2 group to a 27-membered cyclic peptide containing nine amino acids (Duncan et al. 2001). Of all the amino acids, two residues possess nonstandard side chains, and four residues possess D-configuration. Researchers were indulged in total synthesis of chlorofusin; however, synthesis of only 27-membered cyclic peptide core of chlorofusin has been carried out by several groups (Lee and Boger 2009; Wang et al. 2010; Woon et al. 2007).

A second natural p53/MDM2 inhibitor has been obtained from the marine-derived fungal culture—*Arthrinium* sp. The structure of inhibitor hexylitaconic acid was identified using spectroscopic analysis. The inhibition was assessed using ELISA and was found that hexylitaconic acid is inhibiting the interaction with a 50% inhibitory concentration value (IC_{50}) of 50 μg/mL. This is the second inhibitor attained from a natural source (Tsukamoto et al. 2006) (Fig. 7.1a).

Sasiela et al. screened 144,000 natural compounds using a novel high-throughput electrochemiluminescent screen for the identification of inhibitor of MDM2. Authors found semipervine as an inhibitor of MDM2. It was found to inhibit autoubiquitination of MDM2. It also aids in accumulation of p53 in cells owing to its inhibitory action against the process of MDM2-mediated degradation of p53 (Sasiela et al. 2008) (Fig. 7.1b).

Malloy along with his companions performed a bioassay-guided fractionation of two cyanobacterial extracts and isolated hoiamide D. This inhibitor is a polyketide synthase obtained from a natural source that contains two consecutive thiazole and

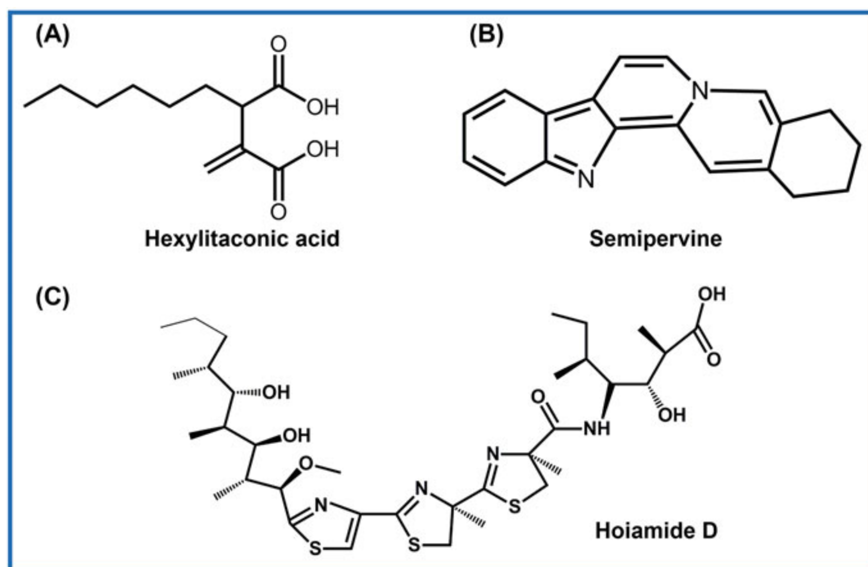


Fig. 7.1 Natural product inhibitors to block P53–MDM-2 interactions: (**a**) hexylitaconic acid, (**b**) semipervine, and (**c**) hoiamide D

thiazolines, and an isoleucine residue with a specific modification. The inhibitor showed inhibitory action against p53/MDM2 with EC_{50} value of 4.5 μM. This implies that it has high potential to serve as an anticancer drug (Malloy et al. 2012) (Fig. 7.1c).

7.2.2 β-Hairpin and α-Helix Peptidomimetics

Fasan et al. have designed β-hairpin protein epitope mimetics as inhibitors targeting p53/MDM2 interactions. Authors marked that the gap amidst the Cα atoms of F19 and W23 in case of MDM2-p53 complex is comparable to the gap among Cα atoms of *i*th and *i* + 2nd residue in case of β-hairpin. This implied that β-hairpin can act as a scaffold to clench the chains of W23 and F19 in the appropriate locations, so that they can interact with binding site of p53 located in MDM2 (Fasan et al. 2004). On the basis of this idea, authors designed a hairpin mimetic in which a loop composed of eight residues was preorganized into β-hairpin by escalating it on d-Pro-l-Pro (dPlP) dipeptide template. It has already been reported that dPlP dipeptide can act as a guide for the stabilization of conformations of β-hairpin loop in cyclic mimetics (Jiang et al. 2000; Shankaramma et al. 2002; Descours et al. 2002). Designed mimetic displayed limited inhibitory action with an IC_{50} value of 125 μM (Fig. 7.2a). Further optimization of this mimetic (Fig. 7.2b) has resulted in significant improvement in its inhibitory action with an IC_{50} value of 140 nM. NMR studies marked that such mimetics are involved in interaction with MDM2 at the site

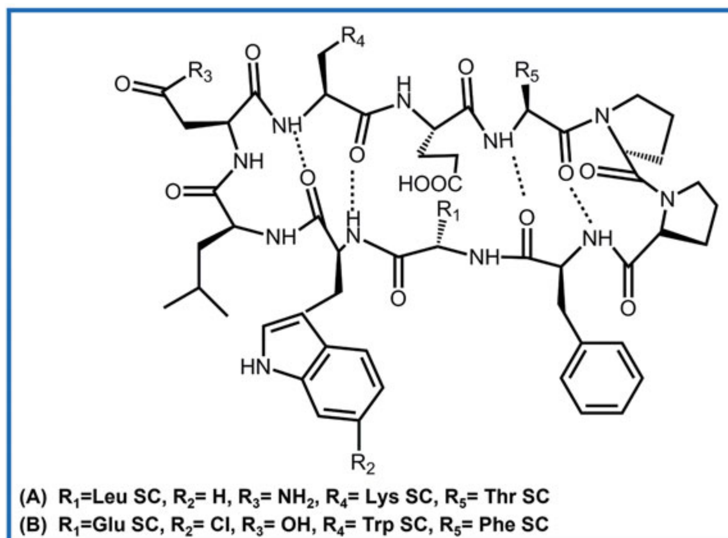


Fig. 7.2 β-Hairpin mimetic designed as inhibitor targeting p53/MDM-2 interactions

which is dedicated for binding p53. NMR-based studies revealed that the previous inhibitor showed less inhibitory activity due to unstable β-hairpin conformation in solution. Further, peptide SAR studies showed that hairpin conformation can be stabilized by introducing aromatic side chain, which could further aid in enhancement of its binding to MDM2 (Fasan et al. 2004). Sang et al. employed sulfono-γ-AApeptides to design inhibitors against p53–MDM2 interactions. Sulfono-γ-AApeptides represent the novel category of unnatural helical foldamers with a potential to block PPIs. They revealed the potential of sulfono-γ-AApeptides to imitate the–helical domain of p53, which can interact with and bind to the hydrophobic groove present in MDM2. Sulfono-γ-AApeptide-based designed inhibitor showed high-binding affinity toward MDM2 with K_d value of 26 nM. It has been shown that sulfono-γ-AApeptides are highly resistant to proteolysis and thus possess an augmented biological activity (Sang et al. 2020).

7.2.3 Terphenyl

Yin et al. have shown that derivatives of terphenyl can imitate one side of α-helical peptide (Yin et al. 2005). If the terphenyl scaffold containing three ortho positions are subjected to substitution by aryl or alkyl groups, the projections of their side chains will correspond to *i*th, *i* + 4th, and *i* + 7th residues of an α-helix. This strategy was employed by the researchers to design terphenyl-based antagonists that imitate α-helical region of p53 and possessing an ability to disturb the MDM2/p53 complex. A small library of terphenyl derivatives were generated and screened using

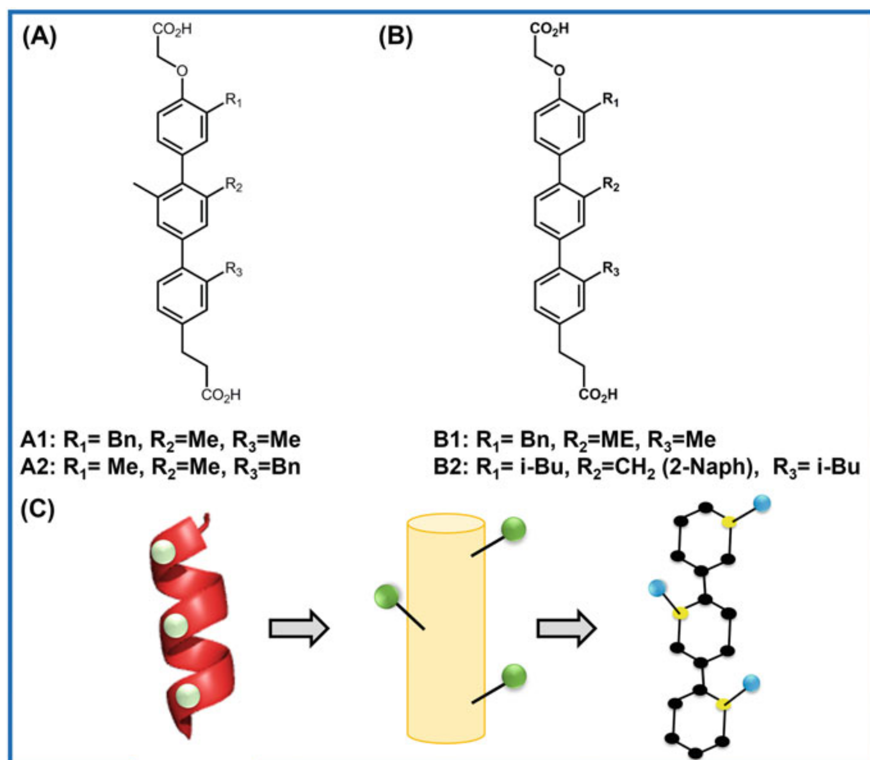


Fig. 7.3 Terphenyl derivatives (**a**, **b**) designed against p53/MDM-2 interactions, (**c**) schematic showing α-helical mimicry based on a terphenyl scaffold

fluorescence-based polarization assay to obtain inhibitors against p53-MDM2 axis (Yin et al. 2005). For terphenyl derivative F7.3A4 that contained isobutyl, 2-naphthylmethylene, and isobutyl side chain sequence, p53 displacement from p53/MDM2 complex was observed with K_i value equal to 182 nM. Terphenyl derivatives F7.3A1 and F7.3A2 which possess reverse side chain sequences showed K_i values 3.83 and 297 μM respectively. This revealed the significance of side chain orientation of terphenyl for binding to MDM2. Improved binding affinities were observed for the terphenyl derivative F7.3A1 and F7.3A3 that contained 2′,6′-‑dimethyl substituents (Fig. 7.3). This improvement is the resultant of enhancement in the rigidity of terphenyl backbone along with lesser entropic debt upon binding to MDM2 (Yin et al. 2005).

Fluorescence experiments strongly recommended that the terphenyl derivatives bind in the same cleft of MDM2, where p53 binds. This binding of terphenyl to the specific cleft was further confirmed using molecular docking and NMR studies. ^{1}H-^{15}N HSQC spectra showed that three amino acid residues (L26, W23, and F19) of p53 interact with three distinct pockets of MDM2. It was further confirmed that terphenyl derivatives also access the same three pockets contained in MDM2, where

the triad residues of p53 interact. Binding modes of terphenyl derivatives to MDM2 were unraveled using molecular docking program (Autodock), which also confirmed that the terphenyl accesses the same surface area on MDM2, as p53 does (Yin et al. 2005).

7.2.4 Nutlins

Vassilev et al. have discovered nonpeptide-based small-molecule inhibitors against p53/MDM2 interactions known as Nutlins. Nutlins belong to cis-imidazoline group of molecules that were found using high-throughput library screening containing synthetic compounds. Nutlins were able to displace p53 from the complex of p53-MDM2 with an IC_{50} value lying in the nanomolar range (100–300 nM) (Vassilev et al. 2004). Nutlins were synthesized as racemic mixtures from which their enantiomeric forms can be separated via a chiral column. As shown in Fig. 7.4, Nutlin 1 (**F7.4A1**), Nutlin 2 (**F7.4A2**), Nutlin 3/3a (**F7.4B1**), Nutlin 4/3b (**F7.4B2**) showed IC_{50} values 0.26, 0.14, 0.09, 13.6 μM, respectively. Nutlins 3a and 3b are enantiomers, of which Nutlin 3a is the active one. Crystal structure for the complex of Nutlin 2-human MDM2 has been obtained at resolution of 2.3 Å. Structural analysis showed that nutlins imitate interactions of p53 with MDM2. Nutlins involve their different groups for interacting differential pockets contained in MDM2. For instance, one bromophenyl group of Nutlin is involved in interaction with tryptophan pocket, its ethyl-ether group interacts with phenylalanine pocket and its other bromophenyl group occupies leucine pocket. Further, NMR studies on the complex of Nutlin 3-MDM2 complex conducted by Podlaski group have also shown concordance with outcomes of X-ray crystallography studies performed on the same complex (Fry et al. 2004).

Structural optimization of Nutlin 3a has resulted in the development of RG7112 that proved to be a potent inhibitor against MDM2. RG7112 also occupies the deep hydrophobic pocket in MDM2 by involving its 4-chlorophenyl groups to fill W23 and L26 pockets, and its ethoxy group sitting in the F19 pocket (Vu et al. 2013; Tovar et al. 2013). RG7112 showed four times higher potency and improved

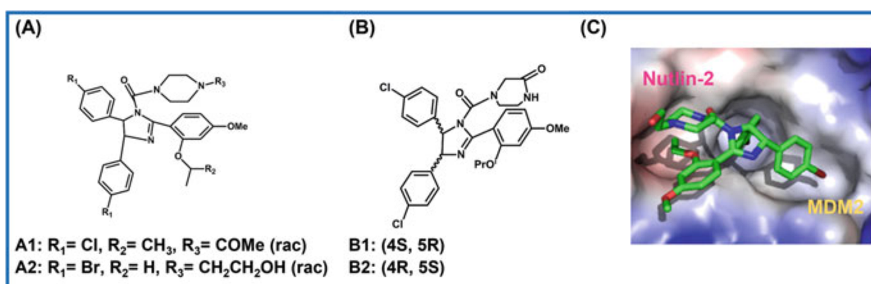


Fig. 7.4 Figure depicting chemical structures of two Nutlin analogs (**a**, **b**); and (**c**) nutlin-2-binding pocket of MDM2

pharmacological features than Nutlin 3a (Tovar et al. 2013). Further, stereochemical modification of pyrrolidine scaffold has resulted in the discovery of second generation of pyrrolidine-based inhibitor RG3788 (Idasanutlin) (Kuusk et al. 2020). It has been reported that although both RG7112 and RG3788 follow the same cellular mechanism, RG3788 shows better activity at lower concentration toward p53 pathways. This is currently under clinical trials to treat solid and hematological tumors (Ding et al. 2013; Kuusk et al. 2020; Kocik et al. 2019).

7.2.5 Benzodiazepines

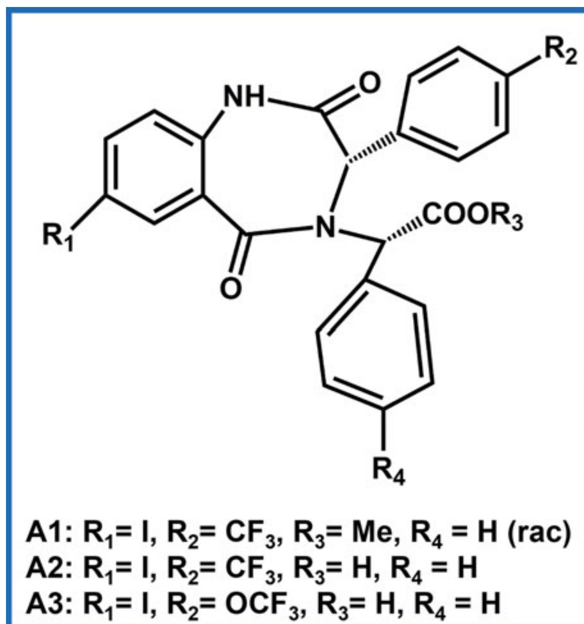
Parks et al. have designed a library of benzodiazepinedione using directed diversity software. High-throughput library screening (HTS) was performed using thermofluor-microcalorimetry to identify novel inhibitors for p53–MDM2 interactions. This HTS assay enables to supervise the unfolding of protein with respect to temperature to screen small molecules that interact and bind to MDM2. Molecules with an ability to bind MDM2 resulting in increase in thermal stability were detected using the thermal denaturation assay. The outcomes of this temperature-based denaturation assay were further verified by measuring change in the T_m values of MDM2 by the addition of peptides that are already known to interact with MDM2. Peptides with high-binding affinity will result in a large shift in the values of T_m. Hits attained using this assay were further confirmed using fluorescence polarization-based peptide displacement binding assay (Parks et al. 2005).

Grasber et al. have employed FP-based peptide displacement binding assay to screen library containing 338,000 compounds and found 1216 positive hits against p53–MDM2 interaction. Amongst the obtained positive hits, 116 hits originated from benzodiazepinedione. One of the hits known as racemic benzodiazepinedione methyl ester is represented in Fig. 7.5. Further, structure optimization-based studies showed that stereochemistry plays an imperative role in the activity of such compounds (Parks et al. 2005). This effort also resulted in the screening of benzodiazepinedione carboxylic acid compounds (**F7.5A2**, **F7.5A3**), which showed low to sub-micromolar potency in a standard p53-MDM2 fluorescence polarization assay.

7.2.6 Spirooxindoles

Ding et al. screened a novel group of nonpeptide inhibitors of MDM2 that are fabricated on the basis of the core structure of spirooxindole. As recommended by the crystal structure of p53/MDM2 complex, the p53/MDM2 interaction is mediated majorly through hydrophobic interactions carried out by F19, W23, and L26. These residues, especially the indole ring of W23 interacts with the hydrophobic cleft present in MDM2. Authors targeted the hydrophobic cleft present in MDM2 for designing inhibitors against p53–MDM2 interaction. NH group of tryptophan is

Fig. 7.5 Figure depicting the chemical structure of racemic benzodiazepinedione methyl ester where A1, A2, and A3 define the combination of different R groups that can be present on the parent structure of the drug



indulged in hydrogen bonding with backbone carbonyl group in MDM2. Hence, chemical moieties imitating interactions of W23 with MDM2 were searched. Substructure search approach was employed to screen the natural compounds containing an oxindole ring. Numerous natural alkaloids such as Alstonisine and spirotryprostatin A containing the core structure of spirooxindole have been identified. It was speculated that these compounds were fitting poorly into the MDM2 cleft; however, the core structure of spirooxindole can be used as an initial point for designing novel MDM2 inhibitors. Oxindole was found to be closely imitating the side chain of W23 of p53 in forming hydrophobic and hydrogen bond interactions with MDM2. Further, two hydrophobic groups that can mimic the side chains of phenylalanine and leucine can also be projected from the scaffold of spiropyrrolidine ring to imitate the side chains of F19 and L26 contained in p53. Candidate compounds were designed using R1, R2, and R3 groups with differential configuration. All the compounds were docked onto the cleft present in MDM2 by employing the GOLD program. In silico molecular docking studies revealed the high-binding affinity of a compound F7.6A1 to MDM2 (Ding et al. 2005). Further, fluorescence polarization binding assay depicted that F7.6A1 and MDM2 interact with a K_i value of 8.5 μM. F7.6A1 was further modified to yield compounds F7.6A2 and F7.6A4. Compound F7.6A4 showed an improved binding with an affinity of 86 nM. Docking studies showed that compound F7.6A4 interacts with MDM2 in the same manner as F19, W23, and L26 in p53 (Ding et al. 2005).

Attempts were made to design novel analogs that can include an additional interaction of L22 in p53 to MDM2. Of all the new analogs, Compound F7.6B1

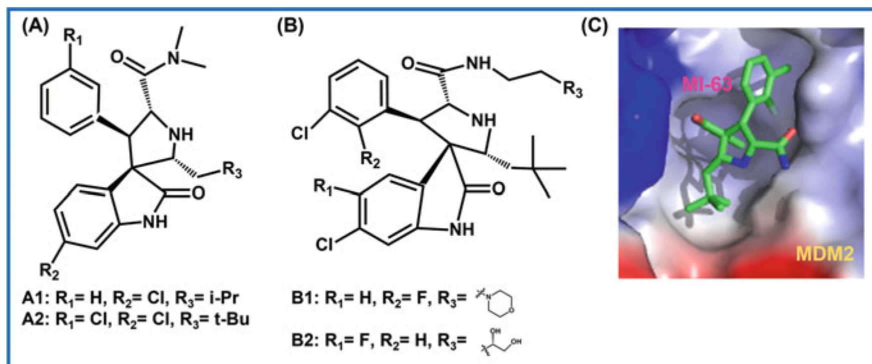


Fig. 7.6 The two analogs of spiroxindole used as p53-MDM2 inhibitors (**a**, **b**). (**c**) The binding pocket of the p53-MDM2 inhibitors

(MI-63) showed K_i value of 3 nM for interacting and binding to MDM2. MI-63 was optimized further to improve its pharmacological properties. The improved version of MI-63 is compound F7.6AB2 (MI-219) which exhibited a binding affinity (K_i) of 5 nM for interaction with MDM2 (Fig. 7.6). MI-219 also displayed desirable pharmacological features and was found to exhibit 10,000-fold more selectivity toward MDM2 in contrast to MDMX. Both these compounds MI-219 and MI-63 displayed an inhibition in the growth of cancer cells by inducing an accumulation of p53. Spirooxindoles MI-219 and MI-63 were the first nonpeptide inhibitors reported with high potency and selectivity (Shangary et al. 2008; Ding et al. 2006).

7.2.7 Chromenotriazolopyrimidines

Allen et al. performed high-throughput screening of a chemical library containing 1.4 million compounds employing homogenous time-resolved fluorescence-based technique. Authors found chromenotriazolopyrimidines (F7.7A1) as inhibitors of MDM2 (Allen et al. 2009). SPR-based binding experiments showed that F7.7A1 interacts and binds with MDM2 with K_d value of 11 μM. Further characterization revealed that F7.7A1 is a racemic mixture containing both syn and trans diastereomers. Of all these forms, only syn form of F7.7A1 possesses an ability to interact with MDM2 with an IC_{50} value of 1.23 μM. During these studies, it was also observed that the compound F7.7A1 is insoluble in water. Crystallized complex of MDM2 and F7.7A1 was analyzed, and it showed that aryl group at seventh carbon of F7.7A1 was occupying the same pocket in MDM2 as occupied by L26 of p53. This aryl group is also involved in interacting with side chain of H96 of MDM2 through weak π-stacking interactions. Aryl group at sixth carbon of F7.7A1 was overlapping with W23 region of p53 while chromene group was involved in interaction with binding pocket employed by F19 of p53. It has been speculated that more efficient compounds can be obtained by optimizing F7.7A1. It was observed that the

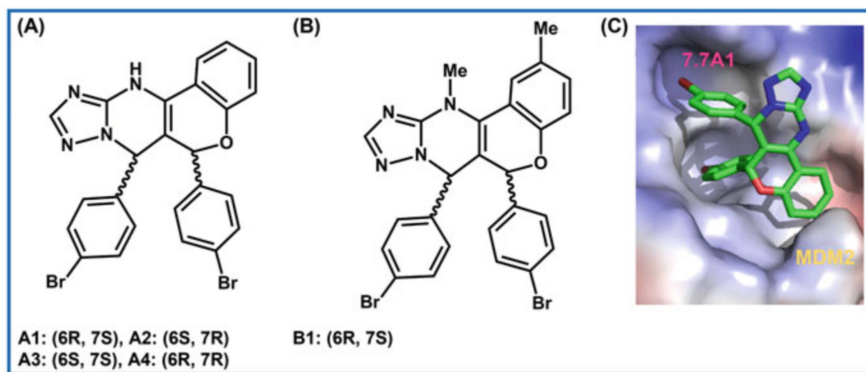


Fig. 7.7 Two analogs of chromenotriazolopyrimidines that are reported as p53-MDM2 inhibitors (**a**, **b**). (**c**) Illustration of the binding pocket of the p53-MDM2 inhibitors

methylation of these compounds results in an increase in the solubility of these compounds and also prevents their racemization. F7.7B1, which is the 6R,7S stereoisomer of F7.7A1 showed high-binding efficacy to MDM2 with an IC_{50} value of 0.39 μM (Allen et al. 2009) (Fig. 7.7).

7.2.8 Piperidinones

Rew et al. have designed novel scaffolds for MDM2 inhibitors using a de novo approach. Their continuous efforts have resulted in disclosure of 1,3,5,6-tetrasubstituted piperidinone F7.8A1 that showed IC_{50} value of 2.42 μM. About 50–70 fold increase in binding potency was observed by the compound F7.8A2, which is the resultant of inversion of stereochemical configuration of C3 acetic acid substituent in F7.8A1 (Rew et al. 2012). Docking studies performed for the complex of F7.8A2 to MDM2 showed that L26 and W23 pockets in MDM2 were occupied by C5m-C1 phenyl and C6m-Cl phenyl groups, respectively. F19 pocket was occupied by cyclopropylmethyl group and carboxylate anions are involved in electrostatic interactions with imidazole side chain of H96 of MDM2 (Fig. 7.8). Significant potency was shown by compound F7.8A1 for inhibition of p53/MDM2 complex formation in both biochemical assay (HTRF, Homogeneous Time-Resolved Fluorescence), neutralization assay, and cell-based assays. Further, structural optimization of series of piperidinone compounds has resulted in F7.82 compound which is highly selective in binding to MDM2. Optimization of F7.8B2 yielded F7.8B3 which showed enhanced potency and improved pharmacokinetic features (Rew et al. 2012). X-ray crystal structure of the complex between MDM2 and F7.8B1 depicted the trans C5 and C6 aryl groups of F7.8B1 have adopted gauche-like orientation when in complex with MDM2. Liao et al. have very well reviewed the development in the era of piperidinone-based inhibitors designed against p53–MDM2 interactions (Liao et al. 2018).

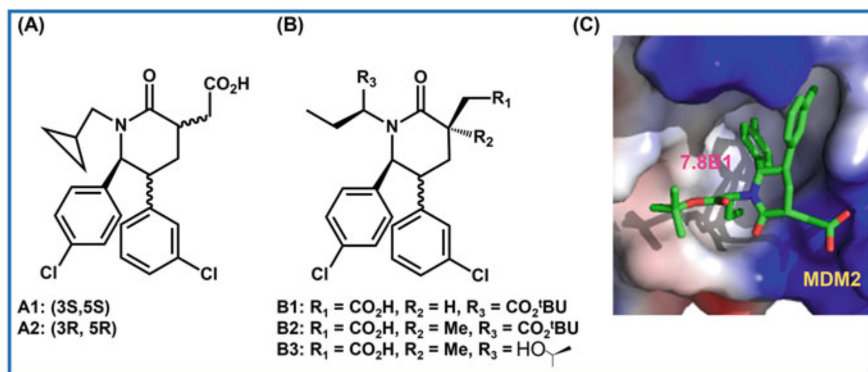


Fig. 7.8 Figure illustrating the analogs of piperidinones that are used as p53-MDM2 inhibitors (**a**, **b**). (**c**) The binding pocket of the p53-MDM2-piperidione inhibitors

7.2.8.1 Indolo-Imidazoles

Furet et al. used the concept of central valine to design nonpeptidic inhibitors of p53-MDM2 (Furet et al. 2012). Based on the structural details about p53/MDM2 interactions, it is suggested that an ideal ligand should efficiently bind to the three sub pockets contained in MDM2. To design such an ideal ligand, it is highly desirable to link three pharmacophore chunks via chemical linkages in a way that it will not add any extra molecular weight to the ligand and also will not disrupt the ligand's interactions with the MDM2 pocket. Molecular modeling attempts have suggested that placement of an aromatic ring which interacts with the V93 side chain via a Van der Waals interaction can provide an appropriate substitution vector for accessibility to three sub pockets. V93 is a residue that occupies the central position in the upper region of MDM2 pocket. On the basis of position of V93, several imidazole-based compounds were synthesized, of which one compound F7.9A showed IC_{50} value of 3.4 μM for inhibiting the p53–MDM2 interaction. Docking studies of F7.9A to form a complex with MDM2 showed absence of one of the hydrogen bonding interactions between the amino and carbonyl group of W23 and L54 of MDM2 respectively. This has driven the authors to replace the chlorophenyl benzylic group of F7.9A with 6-chloroindolyl moiety. This led to the synthesis of compounds F7.9B1 and F7.9B2 that differ from each other possessing aliphatic versus aromatic groups to fill the L26 pocket. While F7.9B1 has two methyl-butyl substitutions to imitate side chain of L26 of p53. F7.9B2 was designed in such a way that it covers the whole sub-pocket. In TR-FRET assay, F7.9B1 and F7.9B2 showed IC_{50} values equal to 0.9 and 0.2 μM respectively.

Efforts were also made to improve the physiochemical properties of these compounds. Solubility was increased by the addition of a solubilizing moiety at second position of indole ring. Since the second position of indole group is accessible to solvent. Hence, synthesis of compound F7.9B3 was carried out and it was tested using TR-FRET assay. F7.9B3 showed IC_{50} values in the micromolar range (0.03 μM). X-ray crystallography studies of complex of F7.9B3 and MDM2 showed

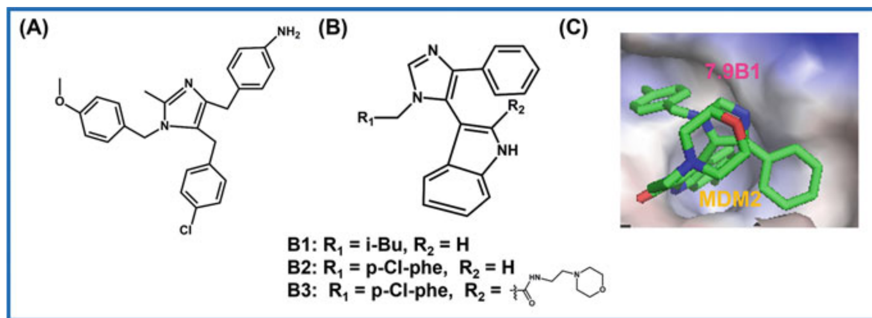


Fig. 7.9 The analogs of indolo-imidazoles that have been used as p53-MDM2 inhibitors (**a**, **b**). (**c**) Illustration of the binding pocket of the p53-MDM2/indolo-imidazole inhibitors

that chlorophenyl ring of F17.5 occupies the L26 pocket while making aromatic stacking interactions with H96 side chain of MDM2. This stacking interaction was found to be advantageous for the inhibitory action of these compounds. It has been suggested that F7.9B2 showed higher inhibitory activity in contrast to F7.9B1 due to the contribution of the stacking interactions (Furet et al. 2012) (Fig. 7.9).

7.3 Modulation of PPIs Associated with Hsp90 Protein

Hsp90 is a heat shock protein that belongs to the molecular chaperone family which participates in providing conformational maturity, stability, and functionality to the client proteins present in the cell (Pearl et al. 2008). Hsp90 is a multifaceted protein which interacts with variety of cellular proteins under stress condition, and thereby either stabilizes and activates the proteins or subjects them to the proteasomal degradation to sustain their structural and functional integrity within the cell. Hsp90 is located in both bacterial as well as in eukaryotic cells. Under normal conditions, Hsp90 constitutes 1–2% of the total cellular protein content (Prodromou et al. 1996). When the cells are under stress conditions due to heat, hypoxia, heavy metals, low pH or presence of toxins or drugs, there is a tenfold rise in the expression of Hsp90. Hsp90 follows the same mechanism of action in both prokaryotes and eukaryotes to regulate the intercellular processes (Frydman 2001).

Hsp90, molecular chaperone family proteins are found in differential compartments of cells including nucleoplasm, cytosol, mitochondria, chloroplast, and endoplasmic reticulum (Krishna and Gloor 2001). Hsp90 actively participates in the process of protein folding of a variety of client proteins. Hsp90 present in cytosol is crucial for the survivability of cell under all the conditions. As functioning of Hsp90 is highly complex, attempts have been made by several groups to map the network of interactions of Hsp90 by employing both mammalian and yeast systems. Such experimental studies have unraveled the functionality of Hsp90 in number of cellular processes and signaling pathways. Further, Hsp90 interactions vary from protein to protein and are assisted by co-chaperones and ATP. Hsp90 acquires

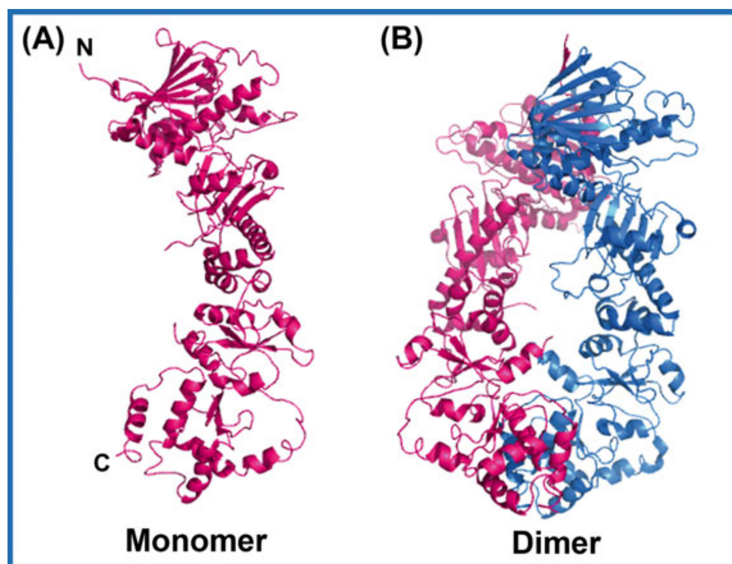


Fig. 7.10 Figure depicting the orthogonal view of monomeric (**a**) and dimeric (**b**) forms of Hsp90 (PDB: 2CG9)

differential conformation when it binds to its client protein (Park et al. 2011a). Hsp90 involves differential site of interactions to interact with differential client proteins (Park et al. 2011b).

Prodromou et al. were the first to solve the structure of N-terminal domain of Hsp90 in 1996 via X-ray crystallography (Prodromou et al. 1996). After a decade, the full-length protein structure of Hsp90 was obtained (Krukenberg et al. 2011). Significant efforts were made to solve the crystal structure of full length of Hsp90 owing to its significant structural flexibility (Krukenberg et al. 2011). It was observed that Hsp90 forms homodimer in its biologically active form. Each Hsp90 monomer contains three main domains that are involved in interactions with other proteins. Monomeric form of Hsp90 contains ATPase domain (25 kDa) at N-terminus, a central/middle domain (55 kDa), and a C-terminal domain (10 kDa) (Fig. 7.10).

N-terminal domain comprises of binding site for ATP and participates in inducing conformational change in the homodimeric Hsp90. This domain is also critical for molecular chaperoning activity. Central domain is involved in interaction with client proteins. It possesses high-binding affinity for co-chaperones, and also exhibits an ability to distinguish between differential substrates (Hawle et al. 2006; Meyer et al. 2003). C-terminal domain is indulged in formation of homodimer and that was established by antibody binding and electron microscopy-based studies. This domain also possesses one ATP-binding region but, the precise role of this domain is still not completely known (Söti et al. 2002).

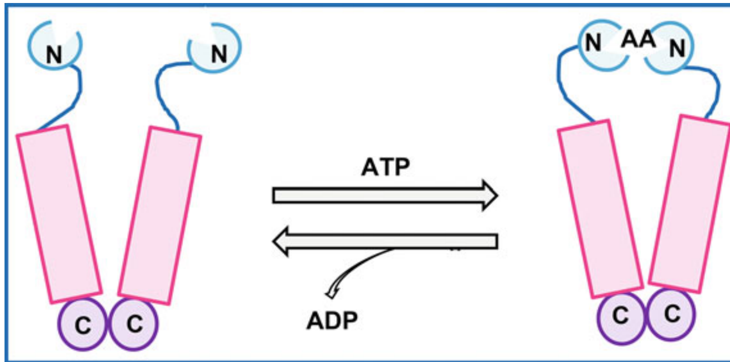


Fig. 7.11 Illustration of ATP-induced conformational changes in Hsp90

Various models have been proposed to provide mechanistic insights about Hsp90 ATPase activity, interactions, and binding with client proteins and ensuring release roles. Conformational changes in Hsp90 are mediated by N-terminal ATP-binding domain. Once the ATP interaction occurs at the domain of Hsp90, it induces the dimerization of Hsp90. This further aids in conformational variability in the amino-terminal and middle domain of Hsp90 in order to construct a mature complex form of Hsp90. This process is imperative to mediate the folding and stabilization of client protein (Shiau et al. 2006; Hao et al. 2010). Upon the accomplishment of Hsp90 function, ATP (present at N-terminal domain) hydrolyzes to yield ADP. This promotes the release of matured protein (Fig. 7.11). If there is any aberration in the folding process, ATP-binding site faces a competition from binding of small molecules to Hsp90, and ultimately the Hsp90 complex undergoes proteasomal degradation (Connell et al. 2001; Kim and Kim 2011).

Hsp90 performs a variety of functions including epigenetic regulation, chromatin remodeling, and signal transduction. Perturbation of Hsp90 results in degradation of the client protein. Under stress conditions, Hsp90 aids in stabilization of client proteins in order to protect the cells from stress. For instance, in cancer cells, many of the oncoproteins act as client proteins for Hsp90 including several kinases such as EGFR, Akt, AR, Raf-1, HER2/ErbB2, and BCR-ABL, transcription factors including HIF1, p53, and others (Sidera and Patsavoudi 2008). Hsp90 is imperative in sustaining the transformation and in increasing the survivability and growth potency of cancer-related cells. Cancer cells require higher activity of Hsp90, as they experience distinct kinds of stress such as hypoxia, acidosis, deficiency of nutrients. Consequently, cancer cells show an upregulated expression of Hsp90 (Nanbu et al. 1996; Mileo et al. 1990; Scaltriti et al. 2012; De Mattos-Arruda and Cortes 2012; Franzén et al. 1997; Gallegos Ruiz et al. 2008; Solit et al. 2003; Li et al. 2008). Hence, blocking the action of Hsp90 may result in inhibition of several oncoprotein clients of Hsp90. It has been proposed that such an inhibition can aid in simultaneous interruption of all the pathways upon which the survival and growth of cancer cells depend (Pearl et al. 2008; Workman et al. 2007). Multitude of

functionalities of Hsp90 makes it a suitable target for designing anticancer drugs (Pearl et al. 2008; Powers and Workman 2007). Inhibitors designed for Hsp90, selectively accumulate in tumor cells in contrast to normal cells; this therapeutic selectivity is due to the predominance of high-affinity active form of Hsp90 in tumor cells as compared to the presence of low-affinity inactive form of Hsp90 in normal cells (Moulick et al. 2011; Kamal et al. 2003).

The extracellular form of Hsp90 is crucial in processes related to cancer cells invasion and for their metastasis. It has been shown that Hsp90 interacts with extracellular domain of HER2/ErbB2, thereby maintains the receptor in active form, which in turn forms heterodimer and interacts with heregulin. The activation of heregulin-induced HER-2 results in downstream signaling to mediate cytoskeletal rearrangement, a prerequisite for cell invasion. This mechanism of action of Hsp90 has been reported for the invasion and migratory activities of breast cancer cells. Hsp90 also assists in activation and maturation of matrix metalloproteinases MMP2 and MMP9, which aids in migration of cancer cells (Eustace et al. 2004; Sims et al. 2011; Stellas et al. 2010). Authors have also demonstrated the participation of Hsp90 in the activation and migration of tumor cells. Cheng et al. reported the interaction of Hsp90 with a cell surface receptor (LRP-1) that activates the signaling process to promote the migration of cells (Cheng et al. 2008). All these findings recommend that blocking the cell surface Hsp90, is beneficial to limit the cancer cell's migration and invasion activities (Collins and Workman 2006; Chen et al. 2005a; Stellas et al. 2007; Li et al. 2012). Taken together all these findings, Hsp90 has evolved as an exciting target for the advancement of cancer therapeutics. Several inhibitors have been designed aiming to target the interactions of Hsp90 with its client proteins (Butler et al. 2015; Goyal et al. 2020; Hu et al. 2018; Kryeziu et al. 2019; Neckers and Workman 2012; Park et al. 2020; Sidera and Patsavoudi 2008, 2014; Wang et al. 2017; Westlake et al. 2019). Some of these inhibitors have been discussed in the following sections.

7.3.1 Inhibitors Targeting N-Terminal ATP-Binding Site of Hsp90

Several inhibitors designed against Hsp90 are under clinical trials. Several reports have depicted that Hsp90 inhibitors as highly effective anticancer agents, with low toxicity, and exerting lesser adverse effects (Sausville et al. 2003; Banerji 2003). Geldanamycin is the first Hsp90 inhibitor that was discovered in 1994. Later, numerous Hsp90 inhibitors were identified.

Two antibiotics including geldanamycin (Fig. 7.12a) and herbimycin (Fig. 7.12a) showed antitumor effect. Studies have reported that geldanamycin (GA) interacts with ATP-binding site present on N-terminal domain of Hsp90, as GA imitates the structure of ATP. The binding of GA to Hsp90 blocks the interaction of ATP with Hsp90, which in turn disrupts the ATP-dependent conformational cyclization of Hsp90 (Stebbins et al. 1997; Prodromou et al. 1997). Interaction of GA and Hsp90 therefore inhibits its chaperone functioning, which subsequently leads to the proteasomal deterioration of its client proteins (Mimnaugh et al. 1996; Stebbins

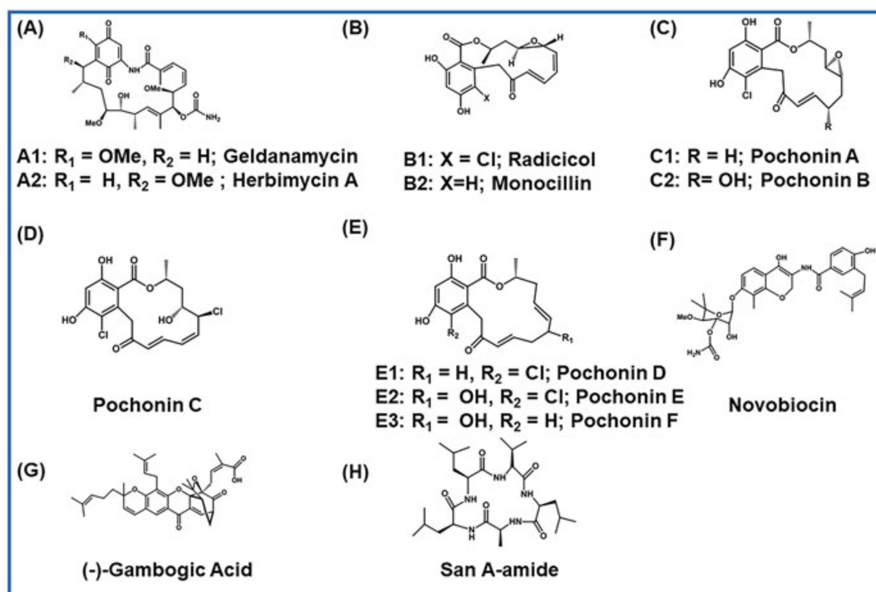


Fig. 7.12 Figure represents various inhibitors targeting N-terminal ATP-binding site of Hsp90 protein. (**a**) Represents root structures of geldanamycin and herbimycin A, where A1, A2 represent the different functional groups present in their structure. (**b**) Root structure of radicicol and monocillin, where B1/B2 denote different functional groups. (**c**–**e**) Represent various types of pochonins. While (**f**–**h**) represent chemical structures of novobiocin, (−)-gambogic acid and san A-amide, respectively

et al. 1997). Structurally, GA encompasses a quinine moiety that is linked to a planar macrocyclic ansa bridge structure between C-16 and C-20. Despite high anticancer potency of GA, it failed to possess clinical potency owing to its lower solubility in aqueous medium, severe hepatotoxicity, and limited in vivo stability as reported by preclinical animal studies (Neckers et al. 1999). This paved the way to design derivatives of GA that retains anticancer potency of GA, along with an improvement in solubility, stability, and toxicological features.

GA analogs containing alkyl amino moiety in place of methoxy moiety at C-17 have displayed reduced hepatotoxicity and better biological activity than GA (Schnur et al. 1995). It has been shown that GA analogs form complexes with Hsp90 in a similar fashion as GA, implicating that the modifications at C-17 position does not affect the interaction of GA analogs with Hsp90 (Tian et al. 2004). GA analogs with modification at C-17 that include 17-allylamino geldanamycin (17-AAG) and 17-desmethoxy-17-*N*,*N*-dimethylaminoethylamino geldanamycin (17-DMAG) have shown low-toxicity profiles than their parent GA as reported by in vivo studies (Fig. 7.13). However, it has been noted that the binding affinity of such analogs for Hsp90 is less as compared to their parental form of GA. Despite showing assuring anticancer effects both under in vitro and in vivo conditions,

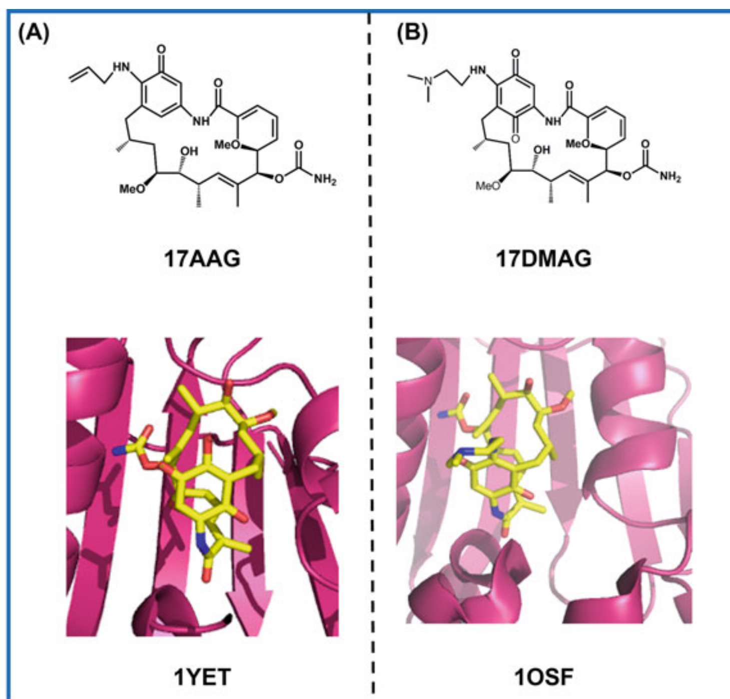


Fig. 7.13 Chemical structures and binding pocket of two geldanamycin analogs (**a**) 17AAG and (**b**) 17DMAG bound to Hsp90

17-AAG possesses lower water solubility. Researchers across the globe have designed the analogs of GA by modifying several groups at different carbon positions (Schnur et al. 1995; Tian et al. 2004; Andrus et al. 2003; Roe, 1999; McErlean et al. 2007; Le Brazidec et al. 2004; Lee et al. 2008b; Schnur and Corman 1994; Eichner et al. 2012) and it has been found that modifications at C-11 position in both GA and 17-AAG have shown augmented cytotoxicity over 17-AAG against a variety of cell lines (Tian et al. 2009). Herbimycin acts in a similar manner as geldanamycin. It has been isolated by Furusaki et al. from the fermented broth of AM-3672, strain of *Streptomyces hygroscopicus* (Furusaki et al. 1980).

Radicicol (RD) belongs to another class of Hsp90 inhibitors (Fig. 7.12b). RD is the most tight inhibitor with an IC_{50} value of 23 nM (Roe et al. 1999). It has been first isolated from the fungi *Monosporium bonorden* and *Monocillium nordini* (Delmotte and Delmotte-Plaquee 1953). Radicicol, a macrocyclic lactone antibiotic, was originally identified as an inhibitor of tyrosine kinase owing to its potential to block the signal transduction pathways associated with oncogenes including Src and K-ras (Soga et al. 2003). Crystal structure analysis of radicicol in complex with Hsp90 depicted that radciciol interacts with the ATP-binding site on Hsp90. It has also been found that RD interacts with Hsp90 with 50-fold higher affinity than GA. Such high affinity of RD for Hsp90 resulted in destabilization of Hsp90 client

proteins. Radicicol does not possess any structural similarity with ATP; however, it interacts with ATP-binding site in a competitive fashion (Roe et al. 1999). RD showed its anticancer effects in vitro but under in vivo conditions, no anticancer effect was observed owing to the metabolic and chemical instability of RD (Proisy et al. 2006). Hence, efforts were made to synthesize analogs of RD. Oxime and cyclopropane derivatives of RD showed anticancer activities, and tolerable toxicity in preclinical animal studies (Proisy et al. 2006; Yamamoto et al. 2003; Soga et al. 2003; Shiotsu et al. 2000).

7.3.1.1 Radicicol

Oxime derivatives of RD including KF25706 and KF55823 showed antiproliferative effects in vitro (Soga et al. 2003). They have also shown antitumor activities in vivo against both breast and colon cancer, without displaying any symptoms related to liver toxicity (Kurebayashi et al. 2001; Agatsuma et al. 2002). Although oxime derivatives of RD showed convincing antitumor effects, none of them have reached clinical trials. In contrast, cyclopropyl derivatives of RD have shown in vitro potentials comparable to that of RD, as they possess improved in vivo potentials (Yamamoto et al. 2003; Yang et al. 2004). Further, hybrid compounds such as Radester (Fig. 7.14a) containing resorcinol and benzoquinone parts from radicicol and geldanamycin, respectively, were synthesized through isopropyl ester linkage. Radester displayed good cytotoxicity against MCF7 breast cancer cells, and was accompanied with the degradation of Hsp90 client proteins including HER-2 and c-Raf (Shen and Blagg 2005). Another hybrid molecule is Radamide (Fig. 7.14b) that contained resorcinol moiety from radicicol and quinone ring from geldanamycin linked through an amide linkage. Radamide was tested against MCF-7 breast cancer cells; it showed inhibition of the ATPase activity of Hsp90, and that was followed by the degradation of Hsp90 client proteins (Clevenger and Blagg 2004). Crystal studies have shown that Radamide, being a hybrid molecule possesses high-binding affinity to Hsp90. This is due to the presence of resorcinol moiety that enables it to bind at the same site where the adenine ring of ADP binds. The presence of quinone

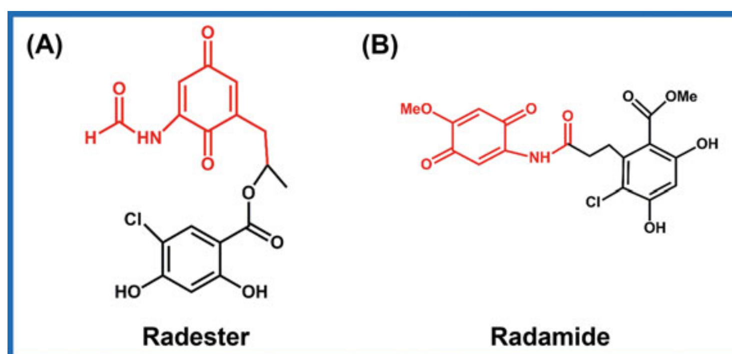


Fig. 7.14 Figure representing hybrid compounds such as (**a**) Radester and (**b**) Radamide made up of GDA and radicicol

ring from GDA enables its binding to the exterior of the pockets which allows its participation in forming hydrogen bonds with amino acids. Docking and molecular modeling studies have shown that Radamide binds appropriately at the ATP-binding site of Hsp90 owing to the presence of resorcinol and quinone moieties (Clevenger and Blagg 2004).

Pochonins A–F (Fig. 7.12c–e), belong to novel class of resocyclic macrolides isolated from *Pochonia chalmydosporia*. Pochonin A is conformationally similar to RD, which showed binding affinity to Hsp90 in the nanomolar range (90 nM). Pochonin C, which is also closely related to RD, is less toxic than RD when used at the same concentrations. Winssinger and coworkers have extensively explored the inhibitory role of pochonins and their analogs (Barluenga et al. 2004, 2005, 2008, 2009; Moulin et al. 2005; Wang et al. 2009).

Several other natural products including Epigallocatechin-3-gallate (EGCG), Novobiocin (Fig. 7.12f), Derrubone, Gedunin, Gambogic acid (Fig. 7.12g), Celastro, etc. have also been reported as inhibitors of Hsp90 (Aeluri et al. 2014). Novobiocin has been reported to bind weekly to C-terminal nucleotide domain of Hsp 90 and induce degradation of Hsp90 client proteins. Several structural analogs of Novobiocin have also been designed which showed 1000-fold higher efficacy against a variety of cancerous cells (Donnelly and Blagg 2008). Gambogic acid obtained from the resin of *Garcinia hurburyl* tree displays the inhibitory activity against Hsp90 (Davenport et al. 2011). It has also been shown that Gambogic acid is able to block the interaction of Hsp70, Hsp90, and CDC 37 with heme-regulated eIF2R kinase, thereby interrupting the maturation of HR1 invitro. Docking studies showed that Gambogic acid interacts with Hsp90 at a position different from that of the ATP-binding site of Hsp90 (Davenport et al. 2011). Hence, it is proposed that binding site of Gambogic acid at Hsp90 can also be used as a target for designing inhibitors against Hsp90 (Davenport et al. 2011).

Sansalvamide A-amide (San A-amide) (Fig. 7.12h) functions as an allosteric inhibitor of Hsp90. San A-amide interacts with the N-terminal domains of Hsp90 and blocks the interaction of proteins specific for C-terminal of Hsp90. San A-amide showed 100-fold better inhibitory activity against blocking the C-terminal binding of the client proteins of Hsp90 in contrast to novobiocin (Sellers et al. 2010; Vasko et al. 2010).

The complexity of natural products and their derivatives faces various limitations in clinical applications, thus promoting the discovery of synthetic inhibitors for Hsp90. Variety of chemical scaffolds including purine, resorcinols, pyrimidines, aminopyridines, azoles, etc. have emerged to serve as inhibitors for Hsp90 (Messaoudi et al. 2011; Sidera and Patsavoudi 2014; Alasia et al. 2012; Blagg et al. 2015; Burlison et al. 2014; Casale et al. 2013; Chimmanamada and Ying 2009; Eggenweiler et al. 2009; Giannini et al. 2010; Hamill et al. 2015; Matulis et al. 2012; Gomez-Monterrey et al. 2012; Moffat et al. 2009; Norrild et al. 2009; Qian et al. 2009; Bussenius et al. 2012; Vukovic and Teofilovici 2012; Yang et al. 2011). Some of the important classes of inhibitors have been discussed briefly in the following sections.

7.3.1.2 Purine Scaffolds

Chiosis et al. designed a small-molecule inhibitor PU3 (Fig. 7.15a) against Hsp90 based on the structural requirements for binding to Hsp90. PU3 showed qualitative inhibitory effects on the expression of proteins, cell proliferation, and differentiation that are highly synonymous to those induced by GM and RD. PU3 showed binding affinity for Hsp90 in micromolar range (15–20 μM). Cells treated with PU3 showed degradation of proteins including Her2 kinase, Raf kinase, and estrogen receptor. PU3 affected those proteins which get affected by RD and ansamycins. Breast cancer cells treated with PU3 showed inhibition in growth, and also displayed significant morphological changes. It has also been observed that the activity of PU3 is less in contrast to natural products. However, the structural scaffold of PU3 can be utilized to design derivatives of PU3 with better activity. Chiosis et al. have also synthesized and characterized a small library of derivatives of PU3. Authors reported that the analog compounds exhibited 30-fold improved cellular effects in contrast to the lead compound PU3, and its derivative compounds with improved binding affinity (0.55 μM) toward Hsp90 have also been identified (Chiosis et al. 2001, 2002).

BIIB021 (Fig. 7.15b) is the first purine-based inhibitor for Hsp90 that entered into the phase of clinical trials. A series of analogs of pyrrole[2,3-d]pyrimidine (deazapurine) have also been designed, of which EC144 (Fig. 7.15c) showed the best blending of pharmacokinetic features and efficacy in murine cancer models (Shi et al. 2012). Hsp90 inhibitors that contained fused amino pyridine core have also been designed for the treatment of Hsp90-related disorders including autoimmune diseases, cancer, and neurodegenerative diseases (Xiong et al. 2008). An oral second-generation inhibitor against Hsp90 known as Debio 0932 has shown promising antitumor activity against wide range of tumors during preclinical studies. PU-H71 is a fully synthetic Hsp90 inhibitor with potential selectivity toward the malignant and normal cells. Studies have also shown potent antineoplastic effects of PU-H71, and specificity toward transformed cells. PU-H71 showed lower binding efficacy toward Hsp90 from normal lung and heart tissues in contrast to that from SKBr3 human breast adenocarcinoma cells. As desired, PU-H71 showed inhibitory

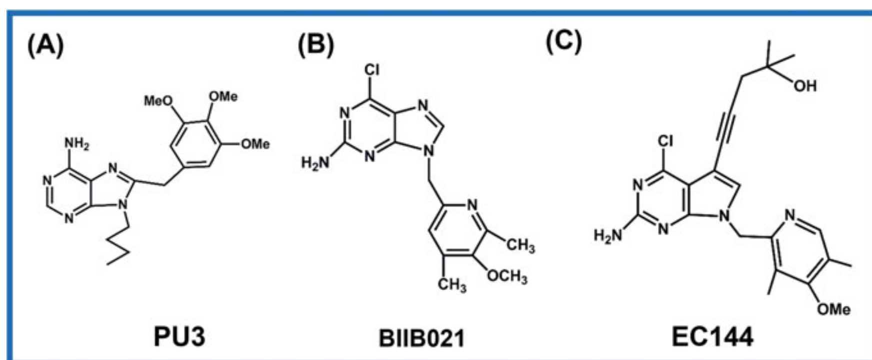


Fig. 7.15 Chemical structures of (**a**) PU3, (**b**) BIIB021, and (**c**) EC144

actions against a number of malignancies, and not hampered the normal cells under the same concentrations (Trendowski 2015).

7.3.1.3 Resorcyclic Pyrazole/Isoxazole Series

Pyrazole represents another class of scaffold that have been identified and modified to develop Hsp90 inhibitors. Cheung et al. performed high-throughput screening of library containing 50,000 compounds and obtained 3,4-diarylpyrazole CCT018159 (Fig. 7.16a) as the most potent Hsp90 inhibitor with an IC_{50} value of 8.9 μM. They have also solved the crystal structure of CCT018159 in complex with Hsp90. It has been observed that CCT018159 binds in the ATPase pocket present at the N-terminal domain of Hsp90 (Cheung et al. 2005). Sharp et al. developed more potent pyrazole amide analogs using structure-based designing approach. For instance, VER-49009 (Fig. 7.16b) and its corresponding isoxazole VER-50589 have been designed, and X-ray crystallographic studies showed that both of them bind in a similar manner as CCT018159. VER-50589 displayed lower IC_{50} (21 nM) values than VER-49009 (47 nM). VER-50589 showed four times better cellular uptake in contrast to VER-49009. Nine-fold better antiproliferative activity against human cancer cell line was observed in case of VER-50589 as compared to VER-49009. These results imply that pyrazole/isoxazole amide analogs possess high therapeutic value (Sharp et al. 2007).

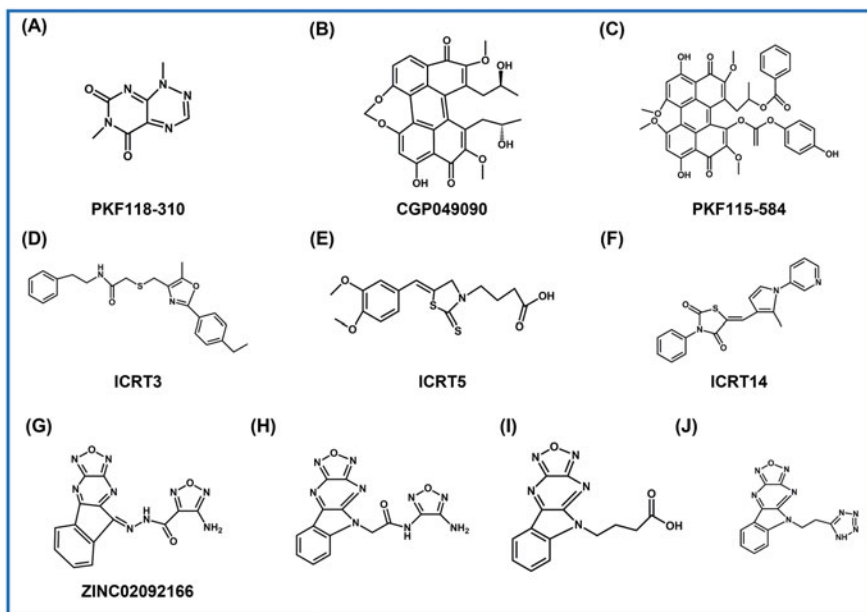


Fig. 7.16 Chemical structures of Hsp90 inhibitors: (**a**) CCT018159, (**b**) VER-49009, (**c**) STA9090, (**d**) STA1474, (**e**) AT13887, (**f**) SNX2122, and (**g**) XL888

STA9090 (Fig. 7.16c), also known as Ganetespib is a resorcinol-containing triazole compound that was shown to block the activity of Hsp90 at a concentration of 10 nM. It has been demonstrated that STA9090 has the potency to disrupt the association of Hsp90 with its co-chaperone p23. STA9090 also showed inhibitory activity against mouse lung adenocarcinomas (Shimamura et al. 2012). Further, a highly soluble prodrug of STA9090, STA1474 (Fig. 7.16d) exhibited excellent inhibitory actions against osteocarcinoma cell lines (McCleese et al. 2009). KW-2478 is another novel resorcinol analog that showed significant inhibition in proliferation of HepG2 cells. It has also been observed that KW-2478 is a vigorous inducer of apoptosis and mediator of cell cycle arrest of HepG2 cells. KW-2478 also inhibited the invasion of HepG2 cells. It has also been marked that at lower concentrations, KW-2478 also promotes HepG2 senescence (Chang et al. 2019).

Woodhead et al. discovered a novel Hsp inhibitor known as AT13387 (Fig. 7.16e), as an outcome of optimization of lead compounds obtained via fragment-based screening approach by Astrex. AT13887 showed antitumor activity against gastrointestinal stromal tumor cell lines at nanomolar concentrations. Kaur et al. have developed a synthetic method to increase the overall yield of AT13387 to assess its clinical utility and for designing its analogs (Woodhead et al. 2010; Smyth et al. 2012; Kaur et al. 2019).

SNX2112 is another novel Hsp90 inhibitor that belongs to the class of benzamides. Assessment of pharmacodynamic and antitumor features of SNX2112 (Fig. 7.16f) by Chandarlapaty et al. showed that SNX2112 is a potent inhibitor of Hsp90. Authors observed that SNX2112 is capable of degrading HER2, mutant version of epidermal growth factor in addition to other client proteins. SNX2112 also inhibited extracellular signal-regulated activation of kinase and Akt and is capable of inducing Rb-dependent G1 arrest followed by apoptosis. SNX5442 is a prodrug of SNX2112 which can be administered orally. After administration, SNX5442 converts into SNX2112, and gets accumulated in tumor cells in contrast to normal cells, thereby leading to the onset of degradation pathways (Chandarlapaty et al. 2008; Huang et al. 2006; Rajan et al. 2011).

Bussenius et al. discovered an orally available Hsp90 inhibitor via a high-throughput screening of 4.1 million chemical compounds that belong to an in-house chemical compound library. Hits were identified using percentage displacement of biotinylated-geldanamycin from the Hsp90 chaperone complex. Screening efforts lead to the identification of 3-amido tropane scaffold-based hits. Hits attained were optimized structurally followed by iterative SAR development process, which resulted in a successful hit known as XL888 (Fig. 7.16g). Assessment of XL888 showed a decline in content of Hsp90 client proteins. Further, efficacy-based experiments conducted in mouse xenograft model (NCI-N87) showed regression of tumor in few dosing recipes (Bussenius et al. 2012). It has been shown that XL888 binds to Hsp90 in differential manner than other Hsp90 inhibitors (Bussenius et al. 2012). XL888 has shown to block the proliferation of numerous human tumor cell lines (Lyman et al. 2011).

Series of carbazole derivatives have been disclosed by the low-throughput screening of library containing 21,000 compounds that were chosen on the basis

of virtual screening (Ruxer et al. 2012). Structure-based designing and chemical synthesis resulted in a compound that showed high potency as Hsp90 inhibitor in several cell-based and biochemical assays including an in vivo assays employing leukemia model (Vallee et al. 2011).

7.3.2 Inhibitors Targeting Hsp90

Marcu et al. assessed the binding of coumarin antibiotics including novobiocin to Hsp90. They found that Novobiocin interacts with Hsp90 at the secondary ATP-binding motif located at C-terminus of Hsp90. Binding of Novobiocin was observed to destabilize the complex of chaperones, release the Hsp90 substrates and co-chaperones along with the degradation of Hsp90 client proteins (Marcu et al. 2000). Attempts have also been made to synthesize the analogs of Novobiocin in order to improve the inhibitory activity of Hsp90 (Marcu and Neckers 2003; Blagg 2009; Blagg et al. 2006). Novobiocin contains three parts including benzamide side chain, noviose sugar, and coumarin core that can be subjected to modification. Library of Novobiocin analogs have been generated, of which a compound showing the degradation of Hsp90 client proteins at a 70 times lower concentration than novobiocin has been identified (Yu et al. 2005).

7.3.3 Inhibitors Targeting Interactions of Hsp90/Co-Chaperone

Hsp90 functions by interacting with a series of chaperones, and by forming a super chaperone complex (Isaacs et al. 2003). Targeting the interaction of Hsp90/co-chaperones to arrest the chaperone cycle and to attain the same inhibitory action as attained in case of directly targeting Hsp90 is another parallel strategy (Gray et al. 2008). This approach of specifically targeting the complex of Hsp90 and co-chaperone represents a more potent and specific way to develop drugs against a variety of cancers (Pearl et al. 2008).

7.3.3.1 Targeting the Cdc37/Hsp90 Interaction

Cdc37 participates in maturation and stabilization of various kinases including nonreceptor tyrosine kinase Src, EGFR, intracellular Ser/Thr kinases (Cdk4 and Raf-1), and lymphocyte-specific protein tyrosine kinase (Lck) (Gray et al. 2008). Cdc37 acts as an adaptor to load these kinases on Hsp90 for the process of their maturation (Vaughan et al. 2006; Silverstein et al. 1998; Pearl 2005). It has been observed that depleting Cdc37 has prevented the coalition of kinase clients with Hsp90, and reduced the levels of these client proteins including Raf-1, Cdk4, Akt, and HER-2/ErbB2, which in turn decreased the proliferation of cells (Smith et al. 2009). This implies that targeting interactions between Cdc37 and Hsp90 marks a therapeutic approach that is highly specific with reduced side effects (Gray et al. 2007, 2008).

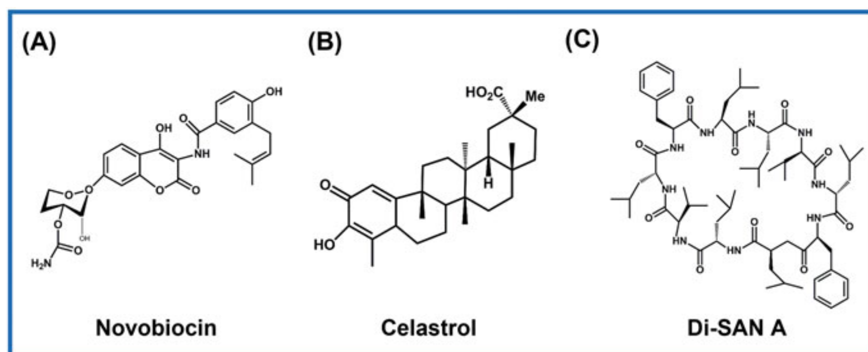


Fig. 7.17 Chemical structures of (**a**) novobiocin, (**b**) celastrol, and (**c**) di-SAN A, that aid in targeting the Hsp90 interactions with its client proteins

Celastrol (Fig. 7.17b) has been isolated from the root bark of Chinese medicine "thunder of god wine" and was discovered as a novel inhibitor/disruptor of interactions pertaining Cdc37 and Hsp90. Celastrol showed antitumor activities in both in vitro and in vivo studies. Both experimental and computational studies including molecular docking and MD simulations confirmed the disruption of interaction between Cdc37 and Hsp90 by celastrol. Authors observed that celastrol induced degradation of Hsp90 client proteins by 70–80%. It has also resulted in elevated expression of Hsp70 protein by 12 times. Authors have also marked that celastrol is able to induce apoptosis in vitro and significantly reduce the growth of tumor. Celastrol also possesses an ability to suppress the metastasis of tumor and serves as a novel inhibitor of Cdc37/Hsp90 complex against pancreatic cancer cells (Gray et al. 2008).

7.3.3.2 Inhibiting the TPR Co-Chaperones/Hsp90 Interaction

Most of the co-chaperones contain TPR (tetratricopeptide repeat) domain that interacts with MEEVD sequence of the carboxy-terminal domain of Hp90 (Pratt et al. 2004; Schopf et al. 2017). Co-chaperones including immunophilins (FKBP51, Cyp40, and FKBP52), Hop/Stil, PP5/Ppt1 possessing TPR domain employ this specific functional domain in order to regulate Hsp90 client proteins. TPR domain comprises of loosely conserved 34 motifs, with 1–16 times repeat in a single domain. They are known to aid in distinct interactions in a variety of cellular processes (Goebl and Yanagida 1991; Blatch and Lässle 1999). Studies have shown that polypeptides exhibiting TPR structural motif possess chaperone-binding function (Moarefi et al. 2003). It has also been elucidated that TPR domains play an important role in assembly of Hsp70/Hsp90 multichaperone complexes (Scheufler et al. 2000). This implies that TPR domains can be used as scaffolds or for designing inhibitors against chaperones. Yi et al. made an effort to design TPR fragment-based inhibitors against Hsp90 using Alpha screen-based high-throughput screening assay (Yi et al. 2009). They found that the molecules retrieved have high potency to be used both as a Hsp90 inhibitor and to understand the activities of co-chaperones in functioning of

Hsp90. Horibe et al. formulated a cell permeable peptidomimetic known as “hybrid Antp-TPR peptide” which is designed on the basis of interfacial interactions between Hsp90 and TPR2A domain of Hop. It has been elucidated that the designed TPR-based peptidomimetic is a potent inhibitor of interactions between Hsp90 and TRP2A domain of Hop. The peptide showed cell death in variety of cancerous cell lines including renal, pancreatic, lung, gastric, and prostate cancer in vitro. It has also been shown that Antp-TPR peptide does not interfere with the viability and the activity of normal cells. In vivo studies showed significant antitumor activity of Antp-TPR peptide against xenograft of human pancreatic cancer in a mice model (Horibe et al. 2011).

Designing inhibitors for the PPIs involving Hsp90 with its specific client proteins provides the ultimate selective approach. However, development of inhibitors based on this approach is limited. This is due to the lack of knowledge with respect to the basis of interaction between Hsp90 and its client proteins (Pearl 2005). Cases in which the crystal data about the complex between the Hsp90 and its client protein are available, the information present about the complex is still deficient to design specific inhibitors (Pearl 2005; Ali et al. 2006; Vaughan et al. 2006).

Plescia et al. have fabricated a cell permeable peptidomimetic known as shepherdin designed on the basis of interaction between Hsp90 and antiapoptotic and mitotic regulator. Shepherdin interacts extensively with ATPase pocket of Hsp90, thereby hampers the stability of Hsp90 client proteins, and leads to death of tumor cells via apoptotic and nonapoptotic means. It has been found that shepherdin has no affect on the survivability of normal cells, as its systemic administration in vivo is tolerable. It is reported to halt the growth of human tumor cells in mice without toxicity. This implies that shephredin is a potent and selective anticancer agent (Vallee et al. 2011). Xu et al. have designed a decoy peptide that disrupts the interaction between Hsp90 and endothelial nitric oxide synthase (NOS). Association of Hsp90 with NOS plays a crucial part in tumor angiogenesis. Hence, these decoy peptides have shown significant inhibition in angiogenesis of tumor and also showed 75% decrease in the growth of tumor when evaluated using mice models (Xu et al. 2007).

Sansalvamide A (San A) is a natural cyclic pentapeptide isolated from marine fungus that has shown antitumor activity in a mid-micromolar range (Belofsky et al. 1999). Studies marked that San A has attained its cytotoxic behavior due to its interaction with Hsp90. Upon interacting with Hsp90, it disrupts the interaction of Hsp90 with specific client proteins including FKBP and IP6K2 (Vasko et al. 2010). Di-San A deca-peptide has been marked to selectively interact and bind to the Middle-C domain of Hsp90, and to disrupt its interaction with IP6K2 at very low concentration (Alexander et al. 2009) (Fig. 7.17c). Further, studies have shown that San-A interacts with Hsp90 at N-terminal and middle domain, and disrupts the binding of Hsp90 with C-terminal-binding client proteins due to the allosteric effect (Alexander et al. 2009). Investigations are going on to figure out the mechanism of action of San-A, and its impact on interaction of Hsp90 with other client proteins.

7.4 Inhibitors Targeting β-Catenin/T-Cell Factor PPIs

Canonical Wnt signaling cascade is imperative in mediating proliferation, differentiation, and communication between the cells (Reya and Clevers 2005; Okamura et al. 1998; Logan and Nusse 2004; Hartmann 2006; Clevers 2006). In the absence of Wnt signal, destruction box mediates an active phosphorylation of β-catenin. The destruction box comprises of proteins including APC (adenomatous polyposis coli), casein kinase 1a, Axin, and GSK-3b. Phosphorylated form of β-catenin undergoes ubiquitination by β-TRCP, (β-transducin repeat-containing protein). When Wnt signal is present, Wnt involves in interaction with frizzled (Fzd)-lipoprotein receptor-related proteins 5 and 6 (LRP 5/6) complex for recruitment of disheveled protein, followed by recruitment of Frz, resulting in phosphorylation of LRP5/6 and recruitment of Axin. This in turn leads to the inactivation of destruction box and lets the inflation of β-catenin in the nucleus. β-catenin present in the nucleus involves in interactions with the member of T cell factor (Tcf)/lymphoid enhancer factor (Lef) family, and leads to the recruitment of transcriptional coactivators including B cell lymphoma 9 (BCL-9), CREB-binding protein (CBP)/p300 (Behrens et al. 1996; Clevers and Nusse 2012; MacDonald et al. 2009). This aids in the translation of Wnt target genes. Additionally, β-catenin also aids in maintaining the shape of the cell, regulation of movement of cell via interaction with cytosolic domains of β-catenin, E-cadherin, and cytoskeleton actin (Molenaar et al. 1996).

Anomalously activated canonical Wnt signaling results in transcription of genes including cyclin D1, c-myc, that are related to proliferation, migration, and survival of cells. Such genes are highly related with the beginning and subsequent progression of numerous cancers including hepatocellular carcinoma (de La Coste et al. 1998), leukemia (Lu et al. 2004), multiple myeloma (Sukhdeo et al. 2007), and colorectal cancer (Tetsu and McCormick 1999; Morin et al. 1997). Cancer stem cells that resist chemo and radiotherapies, can be controlled by signaling from Wnt (Reya and Clevers 2005; Okamura et al. 1998; Logan and Nusse 2004). Hence, disrupting the canonical Wnt pathway is an interesting way to treat cancer. However, it is less desirable to target upstream sites as they can adversely affect the Wnt signaling pathway and can cause perturbation in the functioning of β-catenin in adhesion of cells. It is more beneficial to target the proteins involved in downstream canonical Wnt signaling pathway. Complex formation between β-catenin/Tcf complex represents the penultimate step of the Wnt signaling pathway and can be used as a target for designing inhibitors to overcome several diseased conditions, as the transcription of many genes is dependent on this complex formation (Kim et al. 2002). It has also been shown that removal of β-catenin gene leads to diminish the growth of cancer cells (Kim et al. 2002). Further studies have suggested that it is ideal to target β-catenin in contrast to transcription factor Tcf, as β-catenin is confined to Wnt/β-catenin signaling pathway. In contrast, Tcf participates in multiple signaling pathways (Weber et al. 2011).

Structural studies have been carried out for β-catenin in complex with Xenopus Tcf (Graham et al. 2000), mouse Lef-1 (von Kries et al. 2000), and human Tcf4 (Sampietro et al. 2006; Poy et al. 2001; Graham et al. 2001). It has been found that the typical interaction of β-catenin with Tcf involves a large, flat, featureless

interfacial region. Further, biophysical and biochemical studies also revealed the involvement of three hot spots for the interaction between β-catenin and Tcf (Gail et al. 2005; von Kries et al. 2000). These three hot regions include: (1) interaction region between Lys435/Lys508 of β-catenin and Asp16/Glu17 of Tcf4; (2) interaction domain between Lys312/Lys345 of β-catenin and Glu24/Glu29 of Tcf4; (3) hydrophobic pocket lined with residues including Ile296, Ala295, Phe293, Ile256, Phe253 of β-catenin interacting with Val44 and Leu48 of Tcf4. Binding mode study implies that D16/E17 residues are highly significant than Val44/Leu48 and Glu24/Glu29 of Tcf4 when involved in binding with β-catenin (Fasolini et al. 2003). This study has opened up new avenues to discover novel inhibitors to target the interactions involving β-catenin and Tcf4 (Zhang and Wang 2018).

7.4.1 Inhibitors Designed Against β-Catenin/Tcf4

Lepourcelet et al. reported six small-molecule inhibitors to disrupt the interactions between β-catenin and Tcf by employing SPR (surface plasmon resonance) assay and ELISA (enzyme-linked immunosorbent assay). These compounds showed inhibition with IC_{50} value lying in the micromolar range. Three inhibitors namely 6 (PKF118-310), 5 (CGP049090), and 4 (PKF115-584) (Fig. 7.18a–c), showed

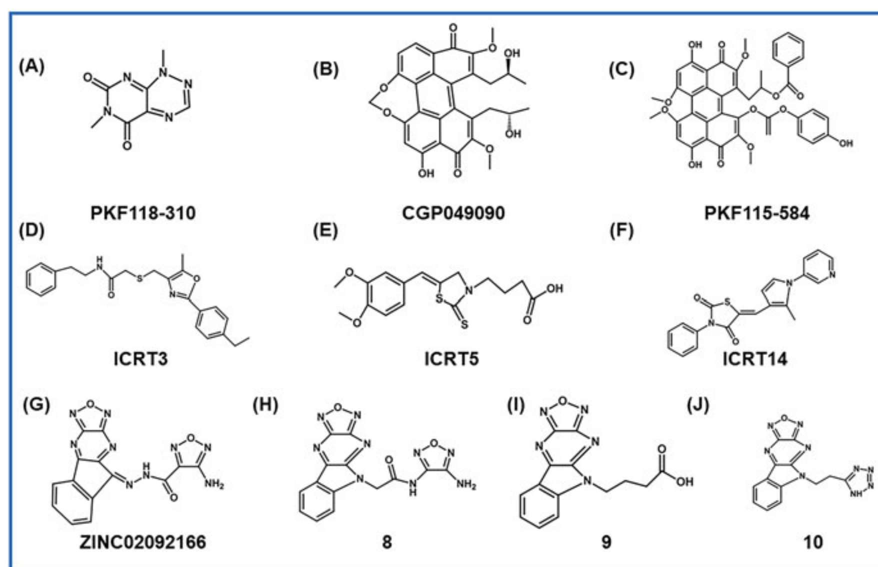


Fig. 7.18 Chemical structures of various inhibitors of β-catenin/Tcf4 interaction: (**a**) PKF118-310, (**b**) CGP049090, (**c**) PKF115-584, (**d**) ICRT3, (**e**) ICRT5, (**f**) ICRT14, (**g**) ZINC02092166, (**h**) inhibitor 8, (**i**) inhibitor 9, (**j**) inhibitor 10

high efficacy to hinder the growth of differential cancer cell types including prostate cancer, colorectal cancer, and multiple myeloma. These compounds have displayed such inhibitory actions both in vitro and in vivo. The actual mechanism of action of such inhibitors is not known completely. However, it is suggested that β-catenin is the major target for these compounds to exert their inhibitory action (Lepourcelet et al. 2004).

Gonsalves et al. took an initiative to design inhibitors that are selective toward β-catenin's nuclear transcriptional function without disturbing its cytoskeletal-related functionality that involves stabilization of adherens junctions located at cellular membrane. Interestingly, the misregulation of β-catenin responsive transcription has been observed in various cancers; hence, it is essential to target β-catenin-mediated nuclear transcription of specific genes. Authors employed RNAi-based modifier screening technique to identify CRT (β-catenin responsive transcription) inhibitors. They scanned a library of 14,977 compounds using high-throughput screening and identified three inhibitors namely, iCRT3, iCRT5, and iCRT14 (Fig. 7.18d–f) (Behrens et al. 1996; Clevers and Nusse 2012; Molenaar et al. 1996). HTS methodology employed Axin-specific dsRNA possessing specificity toward β-catenin interaction with Tcf over other proteins. Identified compounds aimed at blocking β-catenin/Tcf4 interaction showed inhibition in the Wnt target genes expression, and also halted the colon cancer cell growth under both in vitro and in vivo conditions. A pocket containing residues K435 and R469 serves as the major binding site for the iCRT inhibitors. This is due to the reason that these residues are the major contributors providing stability to the β-catenin/Tcf4 interaction (von Kries et al. 2000; Gonsalves et al. 2011).

Catrow et al. designed inhibitors on the basis of acyl hydrazone that serves as an important functional group to discover bioactive molecules. Authors used alpha screening and fluorescence polarization (FP) assays and identified the novel inhibitor designated as ZINC02092166 (Fig. 7.18g–j). This inhibitor showed high inhibitory potency in cell-based assays in contrast to biochemical assays. Further, chemical optimization of this inhibitor resulted in chemically stable inhibitors that showed improved selectivity toward the β-catenin and Tcf interactions. Modes of binding of these inhibitors were discerned using structure-activity relationship-based studies, and site-directed mutagenesis. It has been shown that the positively charged residue R469 of β-catenin, is highly important in binding to these inhibitors. Tetracyclic ring of 12th and 13th of ZINC02092166 was shown to interact via cation–π interaction with guanidino group of R469. It has been proposed that inhibitor 13 can serve as an important initiation point for the fabrication of selective inhibitor against β-catenin/Tcf PPI. Indeed, inhibitor 13 is potential in abolishing Wnt signaling, downregulating the Wnt target genes expression, and inhibiting the cancer cell growth (Catrow et al. 2015).

Using techniques such as virtual screening and biophysical assays (ITC and NMR), Trosset et al. identified the SMIs—14 (PNU74654) (Fig. 7.19a) with a K_d value of 450 nM (Trosset et al. 2006). Tian et al. identified a small-molecule inhibitor designated as BC21 (Fig. 7.19b) by the aid of virtual screening approach in combination with a luciferase reporter assay (Tian et al. 2012). It was found that

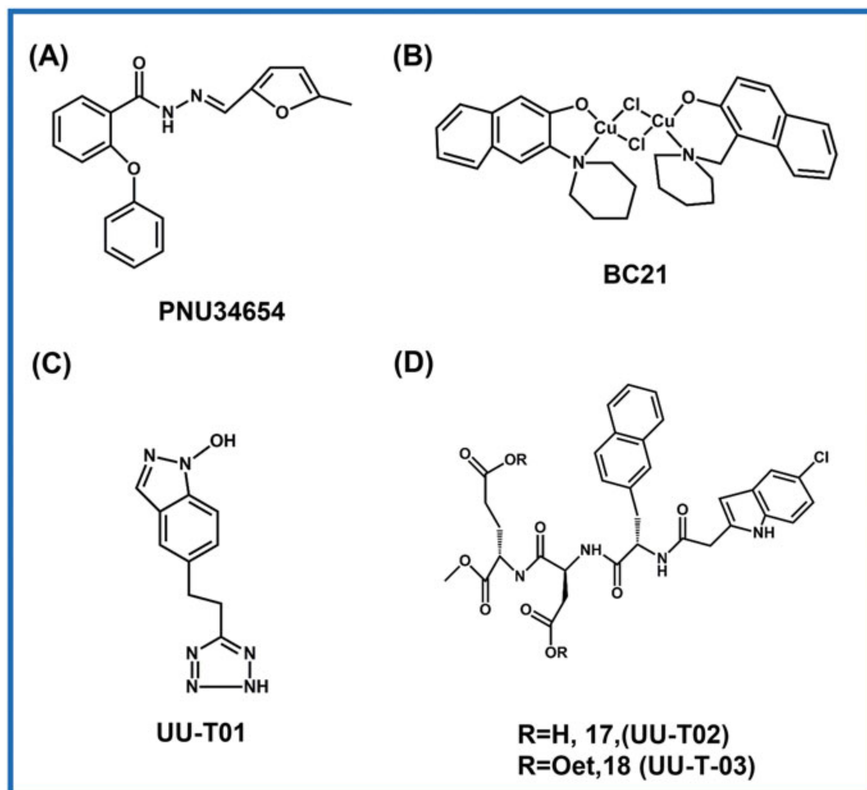


Fig. 7.19 Chemical structures of various inhibitors of β-catenin/Tcf4 interaction: (**a**) PNU34654, (**b**) BC21, (**c**) UU-T01, (**d**) UUT-T02/T03, as described by Trosset et al. (2006) and Tian et al. (2012)

the BC21 binds in a pocket surrounding the positively charged residue K435, and PNU74654 interacts with residues K435/R469 lying in the hot spot region of β-catenin (Trosset et al. 2006). Optimization studies of these compounds (PNU74654 and BC21) have not been carried out, as no experimental studies have yet validated their modes of binding with β-catenin. It is also essential to note that none of these compounds have shown selectivity against the interactions between β-catenin and Tcf over the interaction between β-catenin and other proteins including E-cadherin, APC, etc.

As discussed in Chap. 6, interfacial regions between the interacting proteins contain specific regions/residues that are highly crucial for the interaction and contribute significantly to the binding energies of PPI. Such residues are known as hot spot residues (Clackson and Wells 1995; Guo et al. 2014). Hence, determining such hot spots for the β-catenin/Tcf interaction enables the designing of SMIs on the basis of the structures of hot spots. Studies have found that D16/E17 of Tcf plays an essential role in binding to β-catenin. Hence, D16/E17 is being used as an initial

point for designing inhibitors against β-catenin/Tcf. Yu et al. employed the hot spot-based approach that employs bioisostere replacement to rationally fabricate a nonpeptidic small-molecule inhibitor for β-catenin/Tcf interaction. Authors used bioisostere library to attain novel fragments that can imitate the key binding residues (K435 and K508) of β-catenin (Yu et al. 2013). Efforts of Yu et al. resulted in an inhibitor (UT-T01) (Fig. 7.19c), which binds to β-catenin with a K_d value of 0.53 μM. The binding mode of the inhibitor was verified using SDM and SAR studies. Inhibitor showed specificity toward β-catenin/Tcf interaction in contrast to the β-catenin/APC and β-catenin-E-cadherin PPIs (Yu et al. 2013).

Huang et al. used an alanine-based scanning approach to determine the specific-binding sites that can distinguish the interactions of β-catenin with Tcf, APC, and cadherin, as β catenin/Tcf PPI is responsible for initiation and progression of cancer. In contrast, the interactions of β-catenin with cadherin and APC are involved in cell-cell adhesion and degradation of β-catenin. It was observed that G13 and E17 of human Tcf can be used to design a selective inhibitor for β-catenin/Tcf interaction. Authors used these sites to design UU-T02, a high-binding affinity peptide-based inhibitor for β-catenin (Fig. 7.19d). An ethyl ester derivative of UU-T02, named UU-T03 (Fig. 7.19d) showed high cell permeability and blockage of Wnt signaling pathway along with inhibition in the growth of colorectal cancer cells. UU-T03 was found to inhibit the expression of various proteins lying in the downstream in contrast to those lying at the upstream of Wnt signaling pathway (Huang et al. 2014).

Fang et al. identified a compound known as LF3, a 4-thioreido-benzenesulfonamide derivative via high-throughput screening involving Alpha screen and ELISA techniques. LF3 potentially disrupted the interaction between β-catenin and TCF4. Core structure of LF3 plays an important role in inhibition as revealed by biochemical assays. LF3 showed inhibition of β-catenin signaling pathway in colon cancer cells. LF3 also displayed suppression in features related to Wnt signaling pathway in cancer cells. These features include high motility of cells, progression of cell cycle, and overexpression of Wnt target genes. However, LF3 has not shown any cell death or interference with cadherin-mediated adhesion of cells. Blockage in the self-renewal capability of cancer cells was observed upon treatment with LF3. Further, it has been suggested that LF3 possesses anticancer activity, and is a crucial inhibitor of canonical Wnt signaling. LF3 requires further optimization, preclinical, and clinical studies for its development as novel anticancer agent (Fang et al. 2016). Wang et al. have recently employed structure-activity relationship studies in order to optimize peptidomimetics that are selective against the interactions between β-catenin and Tcf-4 (Wang et al. 2019b).

7.5 Inhibitors Targeting Bcl-2 Family Proteins

Bcl-2 family proteins actively participate in apoptosis. Apoptosis refers to the programmed cell death process, which is imperative for the growth and development of an organ. The process of apoptosis is associated with several biological and morphological changes (Schmitt and Lowe 1999; Silke and Vaux 1998; Lutzker

and Levine 1996; Thompson 1995; Borden et al. 2008). Any aberration in the apoptosis results in different diseases; for instance, an insufficient apoptosis leads to cancer or other autoimmune disorders, and an excessive apoptosis results in Parkinson's or Alzheimer's diseases. Apoptosis occurs via two pathways, the intrinsic and the extrinsic pathways (Hengartner 2000). Extrinsic pathway involves the ligation of cell surface receptors, which in turn results in compilation of DISC (death initiating complex). Intrinsic pathways activate the multidomain proapoptotic Bcl-2 family proteins via a subfamily of Bcl-2 homology (BH)-3 family.

Expression of Bcl-2 protein is involved in hindering the cell death, following multiple pathological and physiological stimuli (Vaux et al. 1988; McDonnell et al. 1989). Bcl-2 proteins are confined to perinuclear membrane, SER (smooth endoplasmic reticulum), and mitochondria (Krajewski et al. 1993; Hockenbery et al. 1990). Apoptosis involves several biochemical events including blebbing of plasma membrane, volume contraction, nuclear condensation, and nucleolytic cleavage of DNA. Bcl-2 present in mitochondria nominates this intracellular organelle to play a significant role during the process of apoptosis (Hockenbery et al. 1990).

Bcl-2 family comprises more than 20 protein members. These proteins are segregated into two classes namely: proapoptotic proteins and antiapoptotic proteins on the basis of their functions. Proteins including Bcl-2, Mcl-1, Bcl-XL, Bcl-B, Bcl-w, Bfl1, and Bcl-A1 belong to the class of antiapoptotic proteins. In contrast, proteins including Bax, Bok, Bad, Bid, Bak, Noxa, BMMD, hrk, Puma are proapoptotic proteins. Among these proteins, Bad, Bid, Bik, Bim, Bmf, Puma, NOXA, and Hrk are known as BH3-only proteins (LI et al. 2018; Moldoveanu et al. 2014). Both category of proteins work synergistically and form dimers to function as "*apoptotic switches*" (Cory and Adams 2002, 2005). Proapoptotic proteins such as Bad and Bax play crucial roles in apoptosis. Binding of antiapoptotic proteins for instance, Bcl-2 to the proapoptotic proteins blocks the functioning of proapoptotic proteins. This implies that inhibiting the interactions between the proapoptotic proteins and antiapoptotic proteins avert tumor cells to elude from the process of apoptosis. Although Bcl-2 family proteins share low-sequence homology, they possess one or four conserved Bcl-2 homology (BH) motifs: BH1, BH2, BH3, BH4 (Moldoveanu et al. 2014). Antiapoptotic subfamily of Bcl-2 proteins comprises all the four BH motifs (Chittenden et al. 1995). These antiapoptotic proteins are involved in suppressing apoptosis. Bak, Bok, and Bax contains three domains including BH1, BH2, and BH3 domains, and are known as "multidomain proteins." Other proapoptotic proteins that are known as "BH3 domain only" proteins contain only BH3 motif. Studies have depicted that BH1, BH2, and BH3 domains play imperative roles in interaction with other proteins of Bcl-2 family; whereas BH4 domain participates in regulating the functions related to cell cycle. Transmembrane (TM) domain is essential for the proper functioning of proteins belonging to Bcl-2 family (Letai et al. 2002; Wang et al. 1996; Kelekar and Thompson 1998; Huang and Strasser 2000; Chittenden et al. 1995).

Structurally, Bcl-2 family proteins contain two hydrophobic α-helices forming a structural backbone and are fenced by amphiphilic α-helices. Hence, there is a formation of an elongated hydrophobic groove or a pocket along the protein surface.

This groove presents a binding site to BH3 domain of proapoptotic protein partners (Petros et al. 2004). Groove is formed by the cleft between the α-3 and α-4 helices, which encompasses a floor formed by α-5 and α-6 helices. The four amphiphilic α-helices forming a hydrophobic pocket in case of Bcl-2 are involved in interaction with Bax (Petros et al. 2004). The homodimer of Bcl-2/Bax is highly stable than Bax/Bak homodimer; hence, Bcl-2/Bax reduces the proapoptotic function of Bax/Bak and thereby prevents apoptosis. Therefore, the lead molecules should be designed to imitate the functionality of proapoptotic protein domains. The optimal lead will interact with hydrophobic pocket present in antiapoptotic protein, and thereby preventing antiapoptotic protein to interact with BH3 domain of proapoptotic protein, thus inducing the apoptosis of cancer cell (Billard 2012; Vogler et al. 2009).

Further, molecular level details of interactions among the Bcl-2 family proteins were deciphered by the structural analysis of a complex between Bcl-XL and a peptide fragment of its interacting protein partner Bak (Sattler et al. 1997). Bak peptide attained an α-helical structure and fitted into the cleft assembled by the two helices of Bcl-XL. The binding cleft of Bcl-XL possessing few sub-pockets was engaged by four branched aliphatic side chains of Bak. Examination of dimensions of binding cleft of Bcl-XL implied that the binding cleft is suitable for binding of drug-like molecules (Fry and Vassilev 2005).

Bcl-2 Family Proteins Participate in Regulating Apoptotic Cell Death

Bcl2 family proteins govern the integrity of outer membrane of mitochondria that is imperative in regulating the process of apoptotic cell death (Reed 1994). Overexpression of many proteins that belong to Bcl-2 family like Bcl-XL, and Bcl-2 have been observed in case of several human cancer types. In order to gain insights into the phenomenon by which the expression of antiapoptotic proteins prevent the cancer cells from apoptosis, it is essential to comprehend how the Bcl-2 family proteins control apoptosis. BH3-only proteins undergo activation in response to death signals such as survival factor withdrawal, DNA damage, and violation of cell cycle check points (Puthalakath and Strasser 2002; Danial and Korsmeyer 2004). The activation of proteins occurs either via means of transcription or via PTM (post-translational modification). Activated BH3-only proteins that include BIM and BID activate BAK/BAX to induce their oligomerization, which results in MOMP (mitochondrial outer membrane permeabilization) (Wei et al. 2000; Desagher et al. 1999; Eskes et al. 2000). This event aids in the discharge of apoptogenic molecules such as DIABLO/Smac, cytochrome C from the intermembrane area of mitochondria. After discharging from mitochondria, cytochrome C interacts with and triggers a complex known as *apoptosome*. Assembly of death-inducing signaling complex (DISC) and apoptosome induces oligomerization and activation of procaspases. Further, activation of caspases occurs via signaling cascade that comprises of an initiator (DISC-activated Caspase 8 and apoptosome-activated caspase-9) and an effector protease (caspase-3), which further induces cellular changes for apoptosis (Green 2000).

Progression of death signal is blocked by Bcl-2 and other antiapoptotic proteins by averting the activation of BAX/BAK. The hydrophobic cleft in antiapoptotic protein interacts with the BH3 domains contained in proapoptotic proteins, thereby leads to heterodimerization of proapoptotic and antiapoptotic proteins (Emily et al. 2001; Cheng et al. 1996). Studies have shown that proapoptotic proteins bind to "BH3-only" proteins more significantly than multidomain proapoptotic protein families. Hence, there is a sequestration of "BH3-only" protein by antiapoptotic proteins following the death signal. This prevents the interaction of "BH3-only" proteins with BAX/BAK, thus impeding the activation of BAX/BAK and MOMP. High expression of proteins that belong to Bcl-2 family including BFL-1, Bcl-XL, MCL-1, have been observed in many cancer types including lung adenocarcinoma, myeloma, etc. (Cuconati and White 2002). Hence, Bcl-2 family proteins serve as valid therapeutic target.

Goal of designing inhibitors to selectively target the interactions of Bcl-2 family proteins and molding their specific pathways in vivo is a daunting task owing to the complexity and size of the interfacial regions among the interacting protein partners. However, technological advancements in computational and experimental strategies have geared up the emergence of novel compounds targeting Bcl-2 family proteins. Several peptides, synthetic small molecules, and natural products have been designed and developed to block the functioning of Bcl-2 family proteins. This is the resultant of continuous efforts made in the direction to understand the detailed mechanisms of their activities/functionalities at molecular level. All the proteins (anti- and proapoptotic) that belong to Bcl-2 family share a similar fold despite differences in their sequence and function, which aids in designing inhibitors for the different proteins that belong to Bcl-2 family. Further, structure-based studies of the complex between Bcl-XL and Bak peptide unraveled the mechanisms related to the formation of heterodimer between the proapoptotic and antiapoptotic proteins (Sattler et al. 1997; Petros et al. 2000). This understanding has further guided the designing of small-molecule inhibitors which specifically target proteins that belong to Bcl-2 family (Garner et al. 2017; Ashkenazi et al. 2017). Some of the molecules designed to interfere with the association and functioning of proteins that belong to Bcl-2 family are discussed in the following sections.

7.5.1 Peptides and Peptidomimetics Targeting Bcl-2 Family Proteins

Chin et al. studied the interactions between the eight "BH3-only" peptides and five Bcl-2 like proteins and found that the occurrence of apoptosis is based on interactions among the specific subset of proteins. The structure of complexes of these proteins provided an initial point for designing mimetics to antagonize the functioning of specific proteins (Chen et al. 2005b). A mutant protein has been designed on the basis of sequence of the NOXA BH3. +ve mutant showed potency to bind both Bcl-XL and Bcl-W2 proteins. Mutant of BIM has also been engineered, and its selective binding for Mcl1 has been shown (Lee et al. 2008a). G3139

(Oblimersen), an 18-mer phosphonothioate antisense oligodeoxynucleotide (ASO) has been designed against the first six codons of Bcl-2 mRNA. ASOs are being used as therapeutic agents with an aim to modulate the expression of a specific gene, as ASO forms ASO/mRNA hybrids to trigger the degradation of mRNA. ASO is stable in in vivo environment owing to its phosphonothioate backbone. Preclinical studies have shown that G3139 downregulates the expression of Bcl-2 protein in vivo in a dosage-dependent and sequence-specific way. In vivo studies marked that the administration of G3139 in humanized mice models with lymphoma showed complete regression of tumor. Further, it was found that the effect of G3139 as a single agent is modest in the cases of solid tumors. However, significant enhancement in antitumor activity was observed when G3139 was used in conjunction with other chemotherapeutic agents including dacarbazine, docetaxel, and anthracycline (Marshall et al. 2004). Peptides have been engineered based on the sequence of BH3 domains of Bcl2 and Bax proteins. Such peptides show high potency for MMP (mitochondrial membrane permeabilization) and induce apoptosis in a manner that Bcl-2 and Bcl-Xl overexpression cannot reverse the process. This peptide-based strategy represented a promising therapeutic approach in the cases of tumors overexpressing Bcl-2 protein (Vieira et al. 2002).

7.5.2 SAHBs (Stabilized α-Helix of BCL-2 Domains)

Using the techniques of hydrocarbon stapling, Walensky et al. designed BH3 peptides with improved pharmacological features. Authors replaced the two nonessential amino acid residues from helical BH3 domain by α, α-di-substituted nonnatural amino acids that contain olefin-bearing tethers in order to design a stapled peptide with all hydrocarbons via ruthenium-based/catalyzed olefin metatheses reaction. Such stapled peptides are known as stabilized α-helix of Bcl2 domains (SAHBs). SAHBs were engineered in a way to imitate BH3 domain contained in BID. As mentioned earlier, BID belongs to the class of proapoptotic proteins possessing BH3 domain only. BID works as an interconnector between the extrinsic and core intrinsic apoptotic pathways. Engineered SAHBs showed helical stability with helical content in the range of 35–87%, in contrast to BH3 domain of BID that contains only 16% helical content in solution. Such helical stability in SAHBs protects it from proteolysis, as it is expected that the helical stability buries the amide backbone. Hydrocarbon stapling in SAHBs also provides protease resistance and stability in serum under both in vitro and in vivo conditions. It has been marked that SAHB activated the apoptotic pathway in order to marshal leukemia cells. Additionally, SAHBs showed significant inhibition in the growth of human leukemia xenografts in vivo (Walensky et al. 2004, 2006). Hence, such stapled peptides with increased half-life in serum and higher cell membrane permeability in contrast to nonconstrained peptides imply that these peptides possess higher efficacy in vivo. Hence, it is suggested that stapling of native peptides via a hydrocarbon represents a promising approach toward modulating PPIs in differential signaling pathways.

7.5.3 Terphenyl

Kutzki et al. designed proteomimetics using terphenyl scaffold in the staggered conformation to reproduce the projections of functionality on the α-helical surface. Authors engineered the mimetics of proapoptotic α-helical peptides to inhibit the interaction of Bak/Bcl-XL. Solution and crystal structure of Bak/Bcl-XL complex were employed to form the basis for the designing strategy. According to the structure of a Bak/Bcl-XL complex, the helical Bak peptide buries into the hydrophobic pocket formed as a resultant of interactions between BH1 and BH3 domains contained in Bcl-XL. Further, alanine scanning experiment clearly showed that hydrophobic residues including V74, L78, I81, I85 that lie on the helical edge are mainly indulged in binding interactions (Wang et al. 2000). Additionally, Asp83 of Bak peptide is involved in formation of an ion pair interaction with lysine residue contained in Bcl-XL. Based on these structural requirements, a series of molecules with terphenyl scaffold encompassing aryl or alkyl substituents on three-ortho positions were formulated. These substituents imitate the important hydrophobic residues lying on the helical exterior of Bak/Bad. Molecules contained substituents of carboxylic acid on both the sides in order to imitate the ion pair interactions. Binding affinity of all the engineered molecules was evaluated using fluorescence polarization assay by involving fluorescein-labeled Bak peptide (16-mer) as a probe. One of designed compound displayed highest binding efficacy with K_d (114 nM) value in the nanomolar range. Docking studies of this compound with Bcl-XL showed that the lead compound is fitting well in the hydrophobic pocket in a similar fashion to that of the Bak peptide. NMR studies depicted that the lead compound binding on the Bcl-XL surface has resulted in the perturbation in the chemical shift values of several residues including A104, Y195, E193, W137, S203, I140, L130, R139, L130 that are lying near the predicted binding site. Perturbed residues belong to the cleft where the helical peptide of Bak binds. Authors have marked that the inhibitor has acquired the same binding pocket as Bak peptide while interacting with Bcl-XL (Kutzki et al. 2002). However, such compounds with hydrophobic and aromatic groups exhibit poor pharmacological features, and are cumbersome to overcome these bottlenecks without compromising their binding affinity (Shaginian et al. 2009). Efforts are being made in the direction to improve their pharmacological characteristics without losing their high-binding affinities for their target molecules.

7.5.4 Natural Products and Their Derivatives

Some of the natural products have also been scanned as modulators of interactions between Bcl-2 family proteins. Some of them are discussed briefly in the following sections (Fig. 7.20).

7.5.4.1 Antimycin A3

Antimycin, isolated from differential *Streptomyces* strains, possesses a unique asymmetrical nine-membered dilactone ring (Lockwood 1953; Liu and Strong

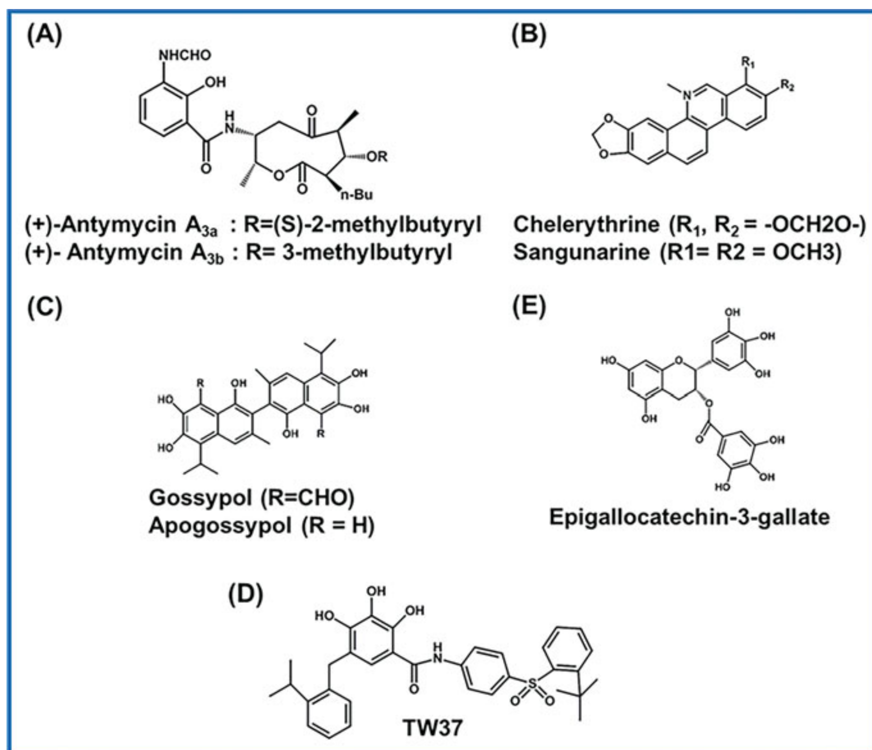


Fig. 7.20 Natural products and their derivatives as modulators of Bcl-2 family protein–protein interactions: (**a**) (+)-Antimycin A3a/A3b, (**b**) Chelerythrine/Sangunarine, (**c**) Gossypol/Apogossypol, (**d**) TW37, (**e**) Epigallocatechin-3-gallate

1959; Kinoshita et al. 1972). Tzung along with his companions demonstrated that an overexpressed Bcl-XL in hepatocyte cell lines showed resistance to many anticancer drugs, but displayed high sensitivity for antimycin A (Tzung et al. 2001; Kim et al. 2001; Kokhan and Shinkarev 2011). Their investigations depicted that antimycin A interacts with Bcl-XL and Bcl-2 in the same manner as a Bak-BH3 peptide (Fig. 7.20a). Authors found that antimycin binds in a competitive manner to BH3 domain of Bcl-XL or Bcl-2 with respect to a BH3 peptide. It has also been reported that antimycin blocked the functioning of Bcl-2 related to pore formation in synthetic liposomes. Binding studies for Bcl-XL carried out using ITC (isothermal titration calorimetry) assay and the intrinsic fluorescence of antimycin showed that antimycin interacts with Bcl-XL with K_d value in the range of 1–2.5 μM. Further, it has also been shown that antimycin interacts and binds in the hydrophobic groove present in Bcl-XL protein in a similar way as BH3 peptide. 2-methoxy antimycin A derivative of antimycin A showed that it preserves BH3-like activity but lacks inhibitory effects on oxidative phosphorylation. Hence, 2-methoxy antimycin can serve as an initial mean of generating anticancer therapeutics targeting Bcl-2 like

proteins (Tzung et al. 2001). Antimycin A3(AA3) includes two enantiomeric compounds namely AA3a and AA3b, that have been prepared by several researchers around the globe.

7.5.4.2 Chelerythrine

Chelerythrine (CHE) (Fig. 7.20b) is a natural benzophenanthridine alkaloid isolated from *Zanthoxylum simulans*. CHE was identified as a displacer of BH3 peptide from Bcl-XL via high-throughput screening assay using fluorescence polarization assay. It has been marked that CHE can directly initiate the cytochrome C release in isolated mitochondria and is capable of inducing apoptosis in cells by efficiently overexpressing Bcl-XL as similar to the control cells. Another CHE-related natural product namely, sanguinarine (SAN) (Fig. 7.20b) showed similar activity as CHE. SAN showed high-binding affinity to Bcl-XL in contrast to CHE. However, the binding of CHE and SAN occurs at different locations in Bcl-XL. CHE binds at BH groove of Bcl-XL, in contrast, SAN binds at BH1 region of Bcl-XL. This implies that both CHE and SAN bind at locations that are different from BH3 peptide-binding site. This indicates that Bcl-XL possesses several regulatory sites that can be accessible to small molecules. NMR studies further confirmed the binding of CHE at BH groove of Bcl-XL, and suggested that chelerythrine initiates the process of apoptosis by targeting the allosteric sites of the proteins that are related to Bcl-2 family (Zhang et al. 2006; Chan et al. 2003).

7.5.4.3 Polyphenolic Compounds

Gossypol (Fig. 7.20c) is a BH3 mimetic obtained from cotton plant. Gossypol has been identified as an inhibitor targeting Bcl-2/Bcl-XL/Mcl-1 interactions during apoptosis in distinct cancer cells (Kitada et al. 2003). Gossypol induces the secretion of cytochrome C and aids in loss of membrane potential of mitochondria without requiring the activation of mPTP and Bak/Bax. Further studies showed that gossypol induces an allosteric variation in Bcl-2 in both Bak−/−/Bax−/− cells and Bcl-2 overexpressing cells. It has also been observed that gossypol aids in significant reduction in the proliferation of tumor xenografts from Bcl-2 overexpressing cells contained in nude mice. In summary, Gossypol possesses an ability to convert an antiapoptotic Bcl-2 into proapoptotic form that results in the cytochrome C release and thereby aids in apoptosis (Lei et al. 2006). HSQC-based NMR studies showed that residues surrounding the hydrophobic groove of Bcl-2 are highly perturbed in the presence of gossypol which indicates that gossypol is involved in interaction with those residues that enable its binding in the hydrophobic groove. Gossypol has been developed as a SMIs of Bcl-XL, Bcl-2, and Mcl-1 proteins. Studies have marked the high toxicity of Gossypol in phase I clinical trials, thereby resulting in its adverse pharmacological features. Presence of two aldehyde groups in Gossypol are accountable for its toxicity. Hence, efforts are being made to reduce the toxicity of Gossypol.

A modified version of natural gossypol known as apogossypol (Fig. 7.20c), which is devoid of both aldehydic groups has been tested. Apogossypol showed its binding activity toward Bcl-2 and Mcl-1 and also retained its cytotoxicity (Wei

et al. 2009). Reduction in systemic toxicity was observed in case of apogossypol as compared to its natural form. Zhan et al. revealed the potency of apogossypol to halt the growth and proliferation of prostate cancer cells by downregulating the expression of Bcl-2 and activation of caspase-8 and caspase-3. Further, in vivo studies demonstrated that apogossypol significantly blocks the growth of tumor in a dose-dependent manner with reduced toxicity in comparison to gossypol. Reports proposed that apogossypol can be further refined as a potential therapeutic agent to treat prostate cancer (Zhan et al. 2015).

7.5.4.4 TW-37

Wang et al. used a structure-based approach to fabricate novel small-molecule inhibitors targeting Bcl-2. They used binding model of gossypol and Bcl-2 to design the structurally new class of compounds to imitate the gossypol/Bcl-2 interactions. Authors found the most potent compound known as TW37 (Fig. 7.20d) that interacts and binds to Bcl-2 with K_i value (290 nM) in the nanomolar range, and also showed high-binding affinities for Mcl-1 and Bcl-XL. TW37 induces cell apoptosis in a dose-dependent reaction and also inhibited the growth of PC-3 prostate cancer cells with an IC_{50} value in the nanomolar range (200 nM). TW37 induces cell apoptosis as a dose-dependent process (Wang et al. 2006; Lea et al. 1993).

7.5.4.5 Epigallocatechin-3-Gallate (EGCG)

EGCG (Fig. 7.20e), is a polyphenolic constituent of green tea. Constituents of green tea are marked for their inhibitory actions on the tumor cell growth (Lin et al. 1996). Leone et al. marked that green tea catechins including black tea theaflavins and EGCG potentially inhibits antiapoptotic Bcl-XL and Bcl-2 by employing the amalgamation of various assays including fluorescence polarization assay, NMR-binding assays, and docking studies. It has been reported that EGCG binds tightly to Bcl-XL. Docking studies depicted that EGCG binds quite well in the BH3-binding pocket present in Bcl-XL (Leone et al. 2003). Structural studies suggested that EGCG and other black tea theaflavins can be improved further via medical synthetic chemistry to increase their selectivity and effective suppression of Bcl-2 family proteins (Leone et al. 2003).

7.5.5 Synthetic Molecules

Several synthetic molecules have also been designed by various research groups across the globe to target Bcl2 family proteins. Some of the molecules are discussed in the following sections.

7.5.5.1 H14

Wang et al. discovered a small molecule known as HA14-1 (Fig. 7.21a), via virtual screening approach. Authors screened a molecular database containing 193,833

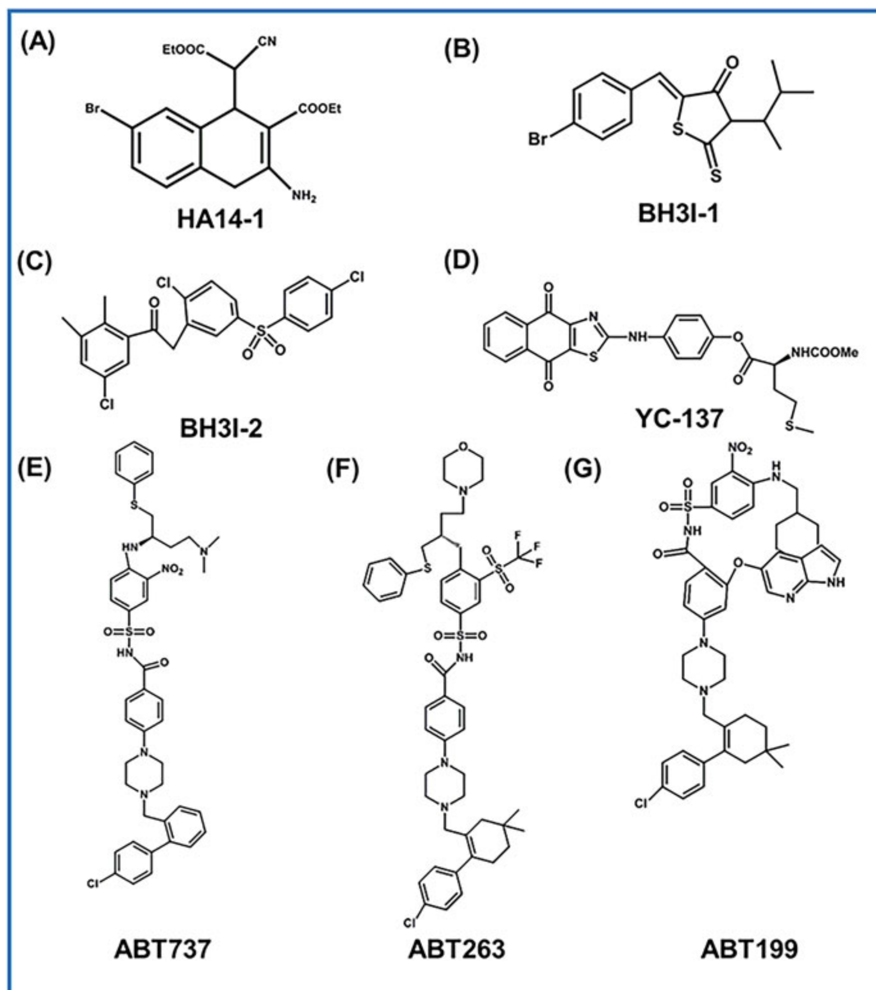


Fig. 7.21 Chemical structures of some of the synthetic molecules targeting Bcl2 family protein interactions: (**a**) HA14-1, (**b**) BH3I-1, (**c**) BH3I-2, (**d**) YC-137, (e) ABT737, (**f**) ABT263, (**g**) ABT199

compounds based on BAkBH3-binding pocket present on the Bcl-2 protein surface. Dock3.5 was employed for screening of the compounds, and the molecules were scored based on the shape complementarity scoring function that imitates Van der Waals attraction energy. Fifty-three compounds were chosen on the basis of favorable shape complementarity, binding energy, and potency to involve in hydrogen bonding interactions with Bcl-2. Of which, twenty-eight compounds were selected for biological testing. Such compounds that exhibit differential scaffolds were

expected to possess drug-like properties. Further, biological testing depicted that HA14 interacts with the Bcl-2 surface pocket, which is imperative for the functioning of Bcl-2. HA14 showed apoptosis in human AML (acute myeloid leukemia) cells that overexpress Bcl-2 protein along with potential reduction in mitochondrial membrane and caspase 9 activation followed by the caspase-3 activation. As HA14 was also found to be cell-permeable, it can serve as probe to study apoptotic pathways regulated by Bcl-2, and can be formulated as a theragnostic agent against cancer (Wang et al. 2000).

7.5.5.2 BH3Is

Degterev et al. screened series of potential SMIs (BH3Is) that can interfere with the interactions of Bak BH3 peptide with Bcl-XL protein. High-throughput screening using fluorescence polarization assay was applied to elucidate that the BH3Is are able to displace Bak BH3 peptide from Bcl-XL. Authors screened around 16,000 compounds contained in a commercial library to identify BH3I-1 (Fig. 7.21b) and BH3I-2 (Fig. 7.21c). The potency of BH3Is to inhibit the heterodimerization of Bcl-XL and proapoptotic Bcl-2 family member protein, tBid was evaluated, and confirmed that BH3Is bind to the hydrophobic pocket of Bcl-XL (Degterev et al. 2001). NMR studies marked that BH3Is resemble proapoptotic BH3 domain, as these compounds exhibit affinity toward BH3-binding pocket of Bcl-XL and occupy the same hydrophobic groove where the Bak BH3 peptide binds (Lugovskoy et al. 2002). BH3Is are also being utilized in optimization of ligands through the combination of computation and structure-based methodologies.

7.5.5.3 YC137

Real et al. formulated and synthesized a small molecule YC-137 (Fig. 7.21d) as an inhibitor of Bcl-2. The role of Y137 was studied in cancer cells and found to be potentially inhibiting a critical interaction between Bid BH3 peptide and Bcl-2. Y137 induced apoptosis in hematopoietic progenitor cells overexpressing Bcl-2. However, Y137 was not able to induce apoptosis in case of breast cancer cells in which there is a significant expression of Bcl-XL and Bcl-2 proteins (Real et al. 2004). Further, studies showed that Y137 is selectively potent against tumors overexpressing Bcl-2 over the tumor cells overexpressing Bcl-XL. It is worth noting that Y137 shows minimal toxicity to normal healthy cells (Xing et al. 2007).

7.5.5.4 ABT-737

Oltersdorf et al. at Abbott research lab explored hydrophobic groove in Bcl-XL and observed the presence of two individual pockets in the hydrophobic groove (Oltersdorf et al. 2005). Authors employed NMR-based high-throughput method known as “SAR by NMR” to identify compounds that can interact with hydrophobic BH3-binding groove contained in Bcl-XL. They obtained two compounds (1 and 2) with K_d value of 0.3 and 4.3 mM, respectively. Based on the analysis of structural data of complexes of Bcl-XL and compounds (1 and 2), researchers generated a highly active lead compound 3 via an addition of a linking group to the compound

2. This new compound 3 showed an IC_{50} value of 36 nM. However, compound 3 showed lower solubility in water and high-binding efficacy toward human serum albumin (HAS). Further, optimization at structural level was carried out to reduce the affinity of compound 3 to HAS via substituting polar groups at specific positions. A compound known as ABT-737 (Fig. 7.21e) was obtained, which showed high affinity toward Bcl-2. ABT-737 interacts with Bcl-2 and Bcl-XL with K_i values of less than 1 and 0.5 nM, respectively. ABT-737 is being widely employed not only to explore the mechanisms related to apoptosis, but also used in preclinical studies of variety of cancers including small cell lung cancer, lymphoma, leukemia (Oltersdorf et al. 2005). However, there is a limitation of poor oral absorption associated with ABT-373 that restraints its clinical application. Bcl-2 antiapoptotic protein inhibitor known as ABT-263 (Navitoclax) has been designed on the basis of structure of ABT-737 (Tse et al. 2008; Park et al. 2008). ABT-263 (Fig. 7.21f) showed its binding to Bcl-w, Bcl-XL, MCL-1, and Bcl-2 with K_i values lying in the nM range. ABT-263 displayed inhibition in the growth of lung cancer xenograft tumor in murine model. Apart from this, ABT-263 coupled with other antineoplastic agents showed synergistic effects to inhibit the growth of blood tumor and solid tumors (Tse et al. 2008). Studies have also revealed that ABT-263 can reduce the platelet count temporarily (Rudin et al. 2012).

A Bcl-2 selective inhibitor known as ABT-199 (Fig. 7.21g) (Venetoclax) has been reengineered on the basis of structure of ABT-263. ABT-199 represents the first small-molecule PPI inhibitor approved to treat chronic lymphoblastic leukemia (Carter and Lazar 2018; Souers et al. 2013). Structural studies of complex between ABT-199 and Bcl-2 have implied that introducing an indole group is helpful in increasing the binding affinity of drugs to P4 pocket of Bcl-2 via hydrophobic interactions, and also facilitate the electrostatic interactions with Bcl-2-specific Asp residues (Souers et al. 2013). ABT-199 showed high-binding affinity toward Bcl-2 with a K_i value of less than 0.1 nM and displayed weak binding affinity toward Bcl-XL ($K_i < 48$ nM). Exquisite inhibitory effects of ABT-199 were observed against acute lymphoblastic leukemia cells. Further, in contrast to ABT-263, both in vivo and in vitro studies evidenced the reduced damage to platelets by ABT-199.

7.6 Inhibitors Targeting 14-3-3 PPIs

Moore and Perez discovered 14-3-3 protein as soluble and acidic protein contained in the mammalian brain (Moore and Perez 1967). The protein is named 14-3-3 as it was obtained in the 14th fraction of bovine's brain homogenate during DEAE cellulose chromatography, and at position 3.3 in starch gel electrophoresis. This protein family exhibits seven isoforms notated by Greek letters α–η (Ichimura et al. 1988). Later, such isoforms were denoted as β, γ, ε, ζ, η, σ, and τ/θ, where δ and α represent the phosphorylated forms of ζ and β respectively (Aitken et al. 1995). These proteins are 25–30 kDa in molecular weight and expressed as conserved regulatory proteins in all the eukaryotic species. 14-3-3 proteins interact with

differential signaling proteins that include phosphatases, kinases, transmembrane receptors. The interactions between 14-3-3 and its target proteins occur by phosphorylated Ser/Thr residues present on the target protein. These residues are located in specific motifs in the target proteins. Such motifs include RSXpS/TXP, RXXXpS/TXP, pS/TX-COOH, where X and pS/T denote any amino acid and phosphorylated forms of Ser/Thr respectively. Interaction of 14-3-3 with its partner proteins depends on phosphorylation of target proteins. Proteins involved in interaction with 14-3-3 include serotonin *N*-acetyltransferase, Raf1 (protein kinase), calmodulin (scaffolding protein), Bad (proapoptotic protein), vimentins, and keratins (cytoskeletal and structure-related proteins). 14-3-3 protein binds with target proteins and results in either inhibitory function like Cdc25 phosphatases or results in stimulatory functions like tumor suppressor p53 protein (Aeluri et al. 2014). Several inhibitors have been designed for the PPIs involving 14-3-3 protein and a few of them are discussed briefly in the following sections.

7.6.1 R18

Wang et al. identified high-affinity peptide-based antagonists that can bind 14-3-3 proteins using phage display libraries. One of the antagonists named as R18 showed high-binding affinity for differential isoforms of 14-3-3. It was observed that R18 binds to ligand-binding groove, which is conserved among all the isoforms of 14-3-3. Mutations at the ligand-binding site in 14-3-3 resulted in drastic decrease in the binding affinity of R18. Binding of R18 to 14-3-3 blocked its binding to Raf-1 kinase and effectively abolished the functioning of 14-3-3. Structure of a complex obtained by cocrystallization of R18 with 14-3-3 ζ deciphered the binding of R18 in the generic-binding groove located in 14-3-3 ζ, thus elucidating the basis for the inhibitory action of R18 on the functioning of the protein. It has been suggested that such peptide-based antagonists not only aid in the development of therapeutic targets, but also assist in understanding the functioning of 14-3-3 in a variety of signaling pathways (Wang et al. 1999).

7.6.2 Exos Macrocyclic Peptide

Glas et al. developed a strategy to prepare macrocyclic peptides encompassing 14-3-3 binding motifs to disrupt the interactions of 14-3-3 protein and its binding protein companions. They formulated the macrocyclic peptide-based inhibitors to block the interactions between the 14-3-3 protein and ExoS. ExoS denotes Exozyme S, a virulence factor from a pathogenic bacterium *Pseudomonas aeruginosa*. Authors designed inhibitory peptides based on the sequence of 14-3-3 involved in binding to ExoS. Inhibitory peptides contained two unnatural amino acid residues that were cross-linked by employing $(CH_2)_n$ bridge. It was found that βss12-(2) inhibitory peptide displayed highest inhibition efficiency. This inhibitory peptide contained S-configured unnatural amino acid residues, which were cross-linked with a chain

constituting 12 methyl groups. Such a macrocyclic peptide showed 30-fold higher binding efficacy for 14-3-3ζ in contrast to the unmodified peptide (nonmacrocyclic). Biophysical studies showed that high-binding affinity of macrocyclic peptides is the resultant of embodiment of cross-links in the peptide that reduced its conformational flexibility. It has been suggested that such strategy of designing macrocyclic peptides can be applied to majority of PPIs involving 14-3-3 protein (Glas et al. 2014).

7.6.3 Tau Epitope

The occurrence of deposition of proteins including amyloid plaques and neurofibrillary tangles (NFTs) is the most striking feature of Alzheimer's disease. NFTs comprise of hyperphosphorylated Tau protein in the form of helical filaments, and also contain ample amount of 14-3-3 proteins (Layfield et al. 1996). It has been observed that 14-3-3 proteins are involved in interaction with Tau protein via phosphorylated Ser214 (pSer214) and Ser324 (pSer324) residues contained in tau protein (Sadik et al. 2009; Sluchanko et al. 2009). pSer214 and pSer324 are the major binding sites for 14-3-3 proteins. Crystallographic structures of 14-3-3 in complex with synthetic peptides composed of phosphorylated pSer214 and pSer324 have been solved and observed that the amino acid sequence (211RTPpSLPTP218) in the vicinity of pSer214 is highly interesting, as it contains three proline residues. More specifically, the presence of Pro218 is unique since such a position has not been occupied by many of the structurally elucidated recognition motifs of 14-3-3 proteins. This provided an inspiration to the researchers to design inhibitory peptides employing P218 position in the Tau peptide to be modified chemically in order to increase the binding efficacy of peptides to 14-3-3 proteins.

Milroy et al. formulated a highly potent inhibitor against 14-3-3 and Tau protein interactions. Inhibitors were engineered on the basis of cocrystal structures of 14-3-3 in complex with a stabilizer (Fusucoccin A) and an inhibitor (based on the Tau epitope sequence). Structures of these complexes showed that there is an overlap between the A ring of Fusicoccin A and Pro218 at C-terminal of a tau epitope. This implied that the extension of C-terminus of Tau epitope with a hydrophobic group will increase its binding affinity to 14-3-3 protein, by allowing the Tau epitope to access a hydrophobic pocket that is well conserved and contained in the amphiphilic groove of 14-3-3. This resulted in three inhibitory peptides namely 109B, 126B, and 201D against 14-3-3 (Milroy et al. 2015). Such chimeric inhibitors encompass conformationally rigid and bulky benzhydryl pyrrolidine moiety at the C-terminal of Tau epitope and showed three times higher binding affinity in contrast to the unmodified phosphopeptide. Further, NMR studies showed the ability of such chimeric compounds to block the interactions of 14-3-3ζ to the full-length phosphorylated form of Tau protein by inhibiting the interactions of phosphoepitope sites present on the C-terminal of Tau protein (Stevers et al. 2017).

7.6.4 BV01, BV02, BV101

Corradi et al. were the first to contribute for a small-molecule inhibitor against PPI between 14-3-3 and c-Abl involved in chronic myelogenous leukemia (CML). Small-molecule inhibitor identified was nonpeptidic in nature. Authors employed the amalgamation of techniques including virtual screening, molecular docking simulations, and structure-based pharmacophore modeling coupled with designing of libraries and synthesis of chemical compounds to screen the inhibitors. They initially screened around 200,000 compounds from ASINEX, a chemical compounds collection and selected 14 compounds that were subjected to further testing using cell-based and biochemical assays. All these efforts led to the recognition of inhibitor known as BV02 (Fig. 7.22a) against the interaction of cAbl and 14-3-3σ (Corradi et al. 2010). BV02 was able to inhibit 14-3-3σ/cAbl complex, and also enabled the nuclear translocation of cAbl at a concentration lying in low μM range in Ba/F3 cells that were expressing wild-type form of Bcr-Abl and its Imatinib-resistant T315I-mutated form (Mancini et al. 2011). Identification of BV02 represented an initial point to develop alternative strategy to treat CML, more specifically for Imatinib-resistant forms. Further, two additional inhibitors known as BV01 (Fig. 7.22b) and BV101 (Fig. 7.22c) were identified using an in silico screening approach against 14-3-3σ/c-Abl PPIs. It was found that BV01 has a potency to elicit apoptotic death of cells expressing Bcr-Abl by blocking the interactions between c-Abl and 14-3-3σ proteins (Corradi et al. 2011).

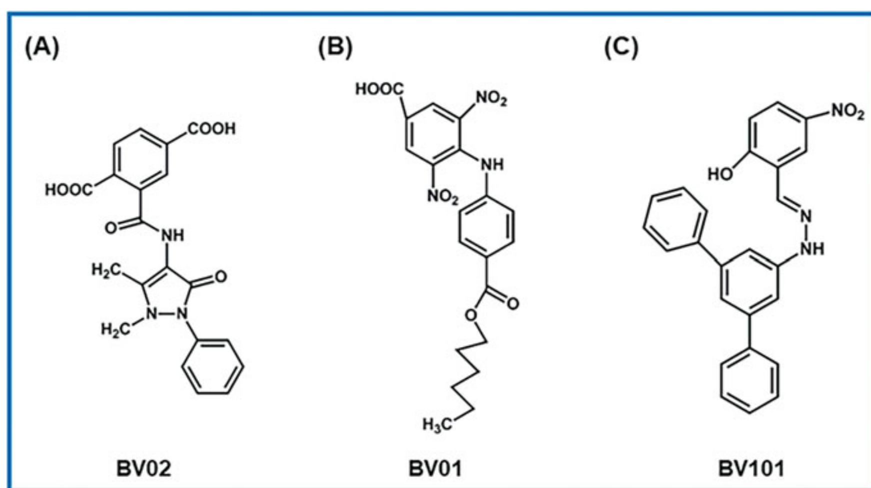


Fig. 7.22 Chemical structures of small-molecule inhibitors: (**a**) BV02, (**b**) BV01, (**c**) BV101, designed to disrupt 14-3-3/c-Abl protein complex

7.6.5 Fobisin

Zhao et al. designed an inhibitor against the 14-3-3γ/p250-Raf-1 peptide interactions by screening large LOPAC (library of pharmaceutically active compounds). Authors scanned this library to obtain hits by employing fluorescence polarization (FP)-based assay. The inhibitor attained was named as fobisin101, a 14-3-3-binding SMI (Fig. 7.23). It was found that fobisin101 is potentially active to halt the interactions between 14-3-3 and Raf-1 or PRAS40. X-ray-derived structure of 14-3-3ζ in complex with Fobisin has been solved with a resolution of 2.39 Å (Iralde-Lorente et al. 2020). The crystal structure encompasses four monomeric units containing two dimers. Each individual monomeric unit comprises of 9 α-helices that are involved in forming amphipathic groove where a client protein binds. Fobisin101 was found to interact with this groove through its pyridoxal phosphate moiety. Exocyclic nitrogen of Fobisin101 was interacting covalently with nitrogen of side chain of K120 and forming a diazene adduct. Phosphate group of Fobisin101 was involved in interactions with K49 and N173 while one face of pyridoxal ring was interacting with I217 through Van der Waals interactions. A solvent molecule was involved in bridging R56 and R127 and 14-3-3ζ involves an array of its basic amino acid residues including R56, R127, and Y128 to interact with phosphate group of phosphorylated ligands. Further, interaction of 14-3-3 with its ligands also involves N173 of 14-3-3 to form intramolecular hydrogen bonding interaction with D114, which in turn is involved in interacting with K120 through salt bridge (Iralde-Lorente et al. 2020). Interaction of 14-3-3 protein with Fobisin and its client protein is similar, implying that Fobisin and its related derivatives can block the interaction of 14-3-3 with its client proteins. Moreover, such pyridoxal-phosphate-based compounds can be employed for both physiological and therapeutic investigations. Further, radiation-based investigations of interactions between 14-3-3ζ and Fobisin

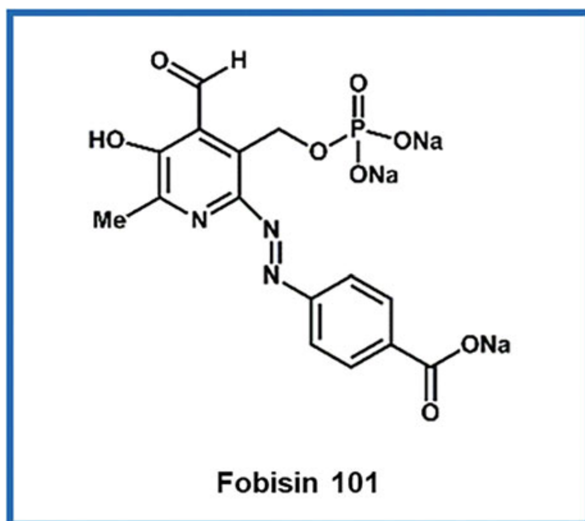


Fig. 7.23 Chemical structure of fobisin101, a 14-3-3-binding small-molecule inhibitor

revealed that this class of molecules can be envisioned as radiation provoked therapeutics to treat 14-3-3-mediated tumor (Zhao et al. 2011). Further, Lorente et al. have contributed two phosphate containing inhibitors namely pyridoxal monophosphate (PLP) and inosine monophosphate (IMP) with an ability to interact with amphipathic groove contained in 14-3-3σ. These molecules possess weak inhibitory action against the 14-3-3/c-Abl protein complex. These compounds are considered as promising initial hits to develop drugs against c-Abl-driven cancers (Iralde-Lorente et al. 2020).

7.7 Inhibitors Targeting c-Myc/Max Interactions

c-Myc is a transcription regulator and a member of the family of basic-helix-loop-helix-leucine zipper (bHLH-ZIP) domain-containing proteins. c-Myc is present in nucleus, where it is involved in regulating the cellular growth, their differentiation, metabolic activities, and death. c-Myc protein interacts with another protein, Max to become transcriptionally active. c-Myc forms heterodimer with Max through the coiling of their bHLH zipper domains. Upon dimerization, complex of c-Myc/Max acts as a master regulator for the transcription process by interacting with Enhancer (E)-box (a specific DNA consensus sequence CANNTG) through its basic region. Binding of this complex results in activation or enhancement of transcription of a regulated gene (Nair and Burley 2003). Functional dysregulation of c-Myc is correlated with numerous human cancer types. c-Myc, encoded by the *myc* proto-oncogene, possesses a highly conserved structure and has found its critical role in augmentation of tumorigenesis, maintenance of tumor cells' growth, their proliferation, differentiation, apoptosis, and angiogenesis (Sabo et al. 2014; Kress et al. 2015; Dang 2012). Abnormally expressed c-Myc has been reported in several cancer types and has been reported as a major driver of cancer. Hence, c-Myc is a major hot spot for the development of therapeutics against cancer (Miller et al. 2012). Efforts are being made in the era of development of inhibitors for the interactions pertaining c-Myc and Max to regulate the expression of oncogenes (Calvis et al. 2021; Ma et al. 2021; Wang et al. 2019a; Singh et al. 2021; Hammoudeh et al. 2009; Follis et al. 2008).

Castell et al. employed BiFC (biomolecular fluorescence complementation) cell-based assay to screen small molecules that can interrupt the interactions between c-Myc and Max. Authors screened potential inhibitory molecules from the library containing 1990 chemical compounds to inhibit c-Myc/Max PPIs. They found three compounds namely: MYCMI-6, MYCMI-11, and MYCMI-14 as potent compounds to block the interactions between c-Myc and Max. Further in vitro experiments and cell-based studies showed that of these three compounds, MYCMI-6 possesses strong inhibitory actions against an interaction between c-Myc and Max. SPR studies depicted that MYCMI-6 blocked Myc-mediated transcription and selectively interacts to Myc bHLH Zip domain with K_d value equals to 1.6 μM. It has been shown that MYCMI interferes with the growth of tumor cells that is dependent on MYC with IC_{50} value lying in the micromolar (0.5 μM) range, while normal cells

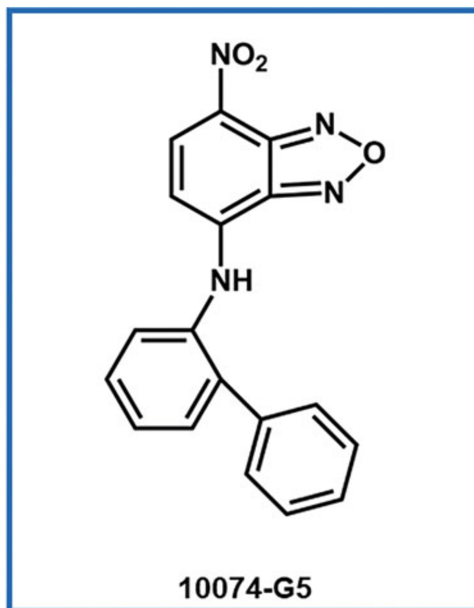


Fig. 7.24 Chemical structure of 10074-G5, which inhibits the formation of c-MYC/Max heterodimer

remain unaffected. Mice xenograft tumor studies marked that MYCMI-6 has a potency to induce apoptosis, to block proliferation of tumor cells, and in reducing the density of microvessels. Further, it has been monitored that MYCMI6 has no effect on the expression of c-Myc, as it specifically targets Myc:Max interaction (Castell et al. 2018).

Chauhan et al. elucidated a SMI—10074-G5 as active disruptor of heterodimerization of c-Myc/Max, as it accomplishes inhibitory function by binding to Myc in its monomeric form (Fig. 7.24). Attempts were made to identify the congener of 10074-G5. Authors discovered a compound known as 3jc48-3 as a congener of 10074-G5. They found that 3jc48-3 interacts with c-Myc five times better (IC_{50} value of 34 μM) than its parental compound. 3jc48-3 possesses twofold selectivity for heterodimers of c-Myc and Max than for homodimers of Max, which implies that the inhibitory action is via interaction with c-Myc. Cell-based studies showed 3jc48-3 inhibits the growth of Daudi cells and c-Myc expressing HL60 cells with IC_{50} values lying in the μM range. Coimmunoprecipitation data analysis suggested that 3jc48-3 blocks the c-Myc/Max complex formation, followed by abrogation of c-Myc-mediated luciferase reporter gene. Half-life of 3jc48-3 was found to be more than 17 h. Altogether, these studies demonstrated high efficiency of 3jc48-3 as an inhibitory target for heterodimeric complex of c-Myc/Max (Chauhan et al. 2014).

Singh et al. have recently discovered a small molecule namely, L755507 through computer-aided drug designing to interrupt the interactions between c-Myc and Max. L755507 has shown to block the growth of cells expressing c-Myc with IC_{50} values lying in the micromolar range. L755507 has also resulted in deteriorating

expression of genes that are the main targets for c-Myc. Further, in silico and spectroscopic-based studies showed that L755507 interacts with c-Myc peptide and mediates stability to its helix-loop-helix conformation. It has been suggested that L755507 can be optimized further as an antineoplastic drug-targeting c-Myc (Singh et al. 2021).

7.8 Inhibitors of KRAS and PDE δ Interactions

Oncogenic RAS is an imperative class of protein that are being employed as targets to design antitumor agents. Proteins that belong to RAS family get modulated in around 20–30% of cancers (Pylayeva-Gupta et al. 2011). RAS family comprises of three member proteins namely: HRAS, NRAS, and KRAS. KRAS is one of the most often mutated proteins in different cancer types. Mutated forms of KRAS have been observed especially in case of pancreas-related pancreatic cancers (Cox et al. 2014). Mutations in RAS proteins make the cells hyperactive to proliferate limitlessly. RAS is involved in regulating the processes including the cellular growth, differentiation, proliferation, and apoptosis of cells. All these functions are mediated by RAS via activating downstream signaling pathways that include PI3K-Akt and MAPK via binding to GTP. In case, RAS proteins remain active, they will continue to interact with downstream effector proteins and transfer signals which in turn result in aberrant proliferation of cells or tumorigenesis (Kang et al. 2004). Hence, RAS proteins can serve as crucial targets to design therapeutics for cancer treatment.

Two methods have been proposed to inhibit the functioning of KRAS protein. One approach involves direct targeting of signaling pathway involving KRAS protein. Second approach involves inhibition of association of KRAS to the membrane. This latter will hinder the KRAS localization in membrane and subsequently halt the signaling pathways augmenting the proliferation of tumor cells. It is required that RAS proteins to be present on the internal layer of the cell membrane to perform its function (Schmick et al. 2014). In order to relocate KRAS to the cellular membrane, recruitment of KRAS toward Golgi apparatus is mediated by its interaction with PDEδ (Chandra et al. 2012; Schmick et al. 2014). This implies that interference with interaction between KRAS and PDEδ, inhibits its localization to the cell membrane, and blocks the signal transduction mediated by oncogenic RAS. However, few studies have reported that the reliability of KRAS on PDEδ is still hazy (Zimmermann et al. 2013). For instance, mice in which PDEδ has been knocked out were fertile, in contrast to the mice in which KRAS has been knocked out resulted in the embryonic lethality (Johnson et al. 1997; Zhang et al. 2007). This implies that the KRS gene is still functionally active even if PDEδ is absent. As the functional dependence of KRAS and PDEδ is ambiguous, obstructing the membrane association of KRAS is the better way to inhibit the functioning of KRAS (Cox et al. 2015). Numerous SMIs to arrest the interactions between KRAS and PDEδ have been identified (Chen and Flies 2013; Chen et al. 2018, 2019; Martín-Gago et al. 2017a; Murarka et al. 2017; Zimmermann et al. 2013, 2014; Kim et al. 2016; Jiang et al. 2017; Zeng et al. 2017).

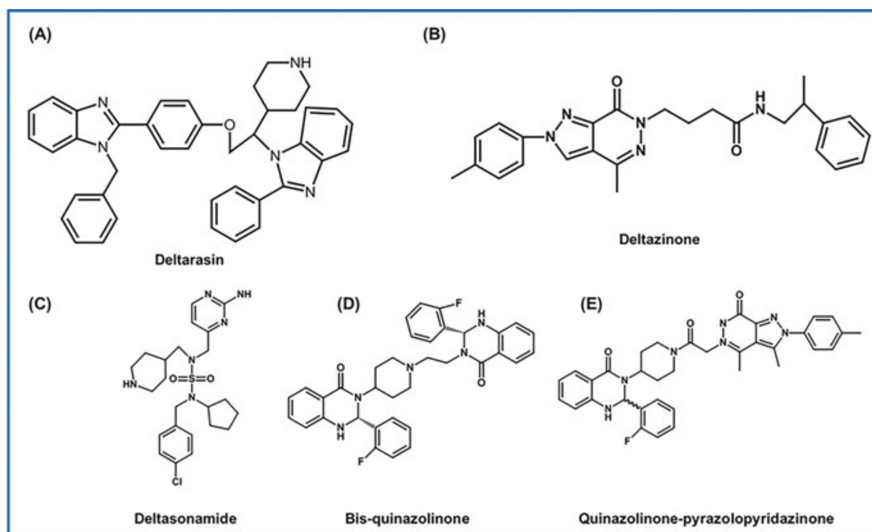


Fig. 7.25 Small-molecule inhibitors (**a**) Deltarasin, (**b**) Deltazinone, (**c**) Deltasonamide, (**d**) Bis-quinazolinone, (**e**) Quinazolinone-pyrazolopyridazinone designed against KRAS/PDEδ interactions

Waldmann and coworkers identified several small-molecule inhibitors including pyrazolopyridazinone inhibitor deltazinone (Fig. 7.25b) (Murarka et al. 2017; Papke et al. 2016), benzimidazole inhibitor deltarasin (Fig. 7.25a) (Zimmermann et al. 2013), and bis (sulfonamide) inhibitor deltasonamide (Fig. 7.25c) (Martín-Gago et al. 2017b) against KRAS/PDEδ interactions. Deltarasin was the first inhibitor to interact with PDEδ with its binding affinity lying in the nanomolar range, but it showed apparent cytotoxicity. Deltazinone and deltasonamide encompass improved binding affinity than deltarasin. However, they showed limitation with respect to their cellular antitumor potencies (Martín-Gago et al. 2017c).

Sheng's group discovered two compounds known as bis-quinazolinone (Fig. 7.25d) and quinazolinone-pyrazolopyridazinone (Fig. 7.25e) as inhibitors of KRAS/PDE δ interactions using the principles of structural biology-based drug designing and virtual screening strategies. Quinazolinone-pyrazolopyridazinone showed moderate levels of antitumor activity against pancreatic cancer cells but displayed high-binding affinity with a K_d value of 2 ± 0.5 nM (Chen et al. 2018). This compound still needs an improvement for its cellular potency.

Further, fragment-based drug discovery approach was used to screen the KRAS/PDEδ inhibitors on the basis of structural details of cocrystallized complexes of PDEδ with fragment-like inhibitors (Jiang et al. 2017; Papke et al. 2016; Zimmermann et al. 2013). Several other novel inhibitory compounds have also been identified by their group to investigate their biological activity and druggability against KRAS/PDEδ interactions. Authors employed SBVS approach and molecular

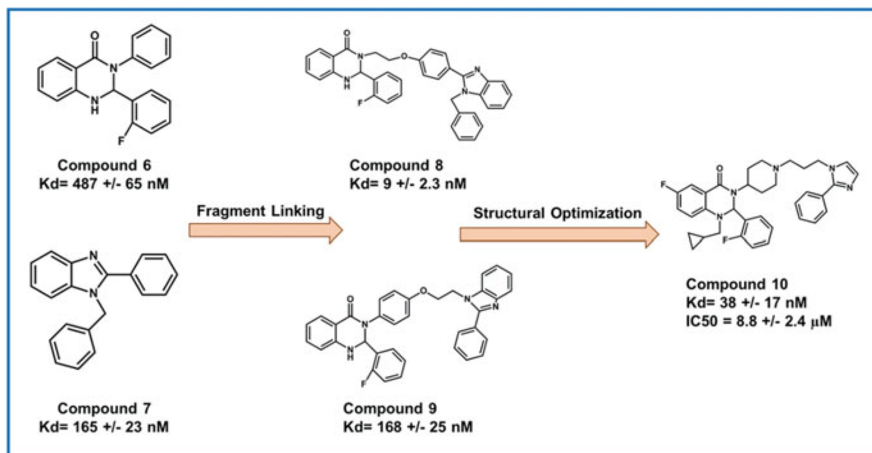


Fig. 7.26 Designing of novel KRAS/PDEδ using fragment-based drug discovery approach

docking approach to screen compound 6 (quinazolinone) and compound 7 (benzimidazole) that possess inhibitory effects against KRAS/PDEδ (Fig. 7.26).

Molecular docking analysis and intermolecular distance measurements of these compounds suggested that fragment linkage is possible at differential sites on these inhibitory compounds. It was found that the distance between the nitrogen of amide contained in compound 6 and benzene ring of compound 7 is 5.3 Å, and the distance between nitrogen atom of imidazole of seventh compound and benzene ring of compound 6 is 5.0 Å. Such distances are suitable to link two methylenes from two compounds via an ether linkage. Linkage of the compounds 6 and 7 in two different ways resulted in two compounds 8 and 9 (Fig. 7.26). Further optimization of these compounds has resulted in compound 10, which showed high-binding affinity toward PDEδ ($K_d = 38 \pm 17$ nM) (Fig. 7.26). Docking-based studies of the complex of compound 10 and PDEδ revealed that cyclopropyl group contained in compound 10 is involved in hydrophobic interactions with I129, L147, and V145 in PDEδ. Compound 10 also showed inhibitory actions in Capan-1 cells by significantly inducing apoptosis, downregulating EFG-induced phosphorylation of Erk and Akt in these cells (Chen et al. 2018).

7.9 Inhibitors of CD40 and CD40L Interactions

T-cells are the crucial players of the immune system. T-cells' stimulation not only depends on the direct provocation from external antigens, but it also requires a transmission of co-stimulus signal generated as a result of interaction of several surface molecules (Chen and Flies 2013). CD40/CD40L pathway is imperative to activate T-cells as any aberration in this pathway leads to a pathological state. CD40, present on the membrane surface participates in the development and activation of

the B-cells. CD40 also serves as surface antigen for the functioning of B-cells and T-cells (Van Kooten 2000). CD40L is an activating molecule that activates both B-cells and T-cells, and is majorly expressed on the surface of T-cells, more explicitly on CD4+ T cells (O'Sullivan and Thomas 2003). Both CD40 and CD40L form a complementary protein pair. CD40 belongs to the class of superfamily of tumor necrosis factor (TNF) receptor. In contrast, CD40L, which is also represented as CD154, is a member of TNF family (Meabed et al. 2007). Both CD40/CD40L protein pairs are mainly expressed by both T-cells and B-cells. CD40/CD40L are the pair of membrane proteins engaged in a variety of physiological functionalities including activation of B-cells, their proliferation and differentiation, production of antibodies, apoptotic processes, activation of T-cells, production of cytokines, cellular immunity, humoral immunity, and inflammation-related activities (Elgueta et al. 2009). Anomalous CD40/CD40L expression is associated with various immune-deficient, cardiovascular, and autoimmune diseases (Senhaji et al. 2015; Pamukcu et al. 2011; Wagner et al. 2002; Elgueta et al. 2009; Bosmans et al. 2021). Such diseases can be treated by interfering with the interactions between CD40 and CD40L.

Several antibodies have been designed that are capable of blocking the interactions of CD40/CD40L. Some of them including dacetuzumab, lucatumumab, bleselumab, etc., have reached the levels of preclinical and clinical stages (Croft et al. 2013). Dacetuzumab, a IgG1-humanized anti-CD40 monoclonal antibody, which activates the proliferation of B-cells, when IL-4 and CD40L are absent. However, it obstructed the proliferation of highly differentiated form of B-cells. Dacetuzumab also aids in ADCC (antibody-dependent cell-mediated cytotoxicity) by transmitting the apoptotic signal via caspase-3 (Oflazoglu et al. 2009). Trials of most of such antibodies have been discontinued owing to their severe thrombolytic side effects (Schulze-Neick et al. 2004; Boumpas et al. 2003; Kawai et al. 2000). In order to circumvent such severe side effects, efforts are being made in the direction of designing small molecules to interfere with CD40/CD40L interactions (Margolles-Clark et al. 2009b, 2010).

Margolles-Clark et al. have reported suramin as an effective inhibitor of CD40/CD40L interactions. Authors found that suramin inhibits the binding of both murine and human CD40L to their cognate CD40 receptor. Suramin also prevented the proliferation of human B-cells mediated by CD40L, and also averted the expression of CD40, CD80, CD84, and MHC class II molecules in a concentration-dependent way. Further, it has also reduced the release of interferon gamma, IL-6, and IL-8. Suramin has also been marked to block the interaction of TNF with its receptor. However, it has been speculated that suramin inhibits the interaction of CD40/CD40L 30 times better than that to the interaction of TNF with its receptor. Suramin possesses multiple pharmacological effect, which limits its clinical usage for the treatment of diseases like AIDS and cancer (Margolles-Clark et al. 2009a).

Margolles-Clark et al. have also investigated the efficiency of small-molecule organic dyes to interfere with the interactions of CD40/CD40L (Fig. 7.27). Small-molecule organic dyes were found to be significantly active in halting the CD40/CD40L interactions in a concentration-dependent manner. Their IC_{50} values were

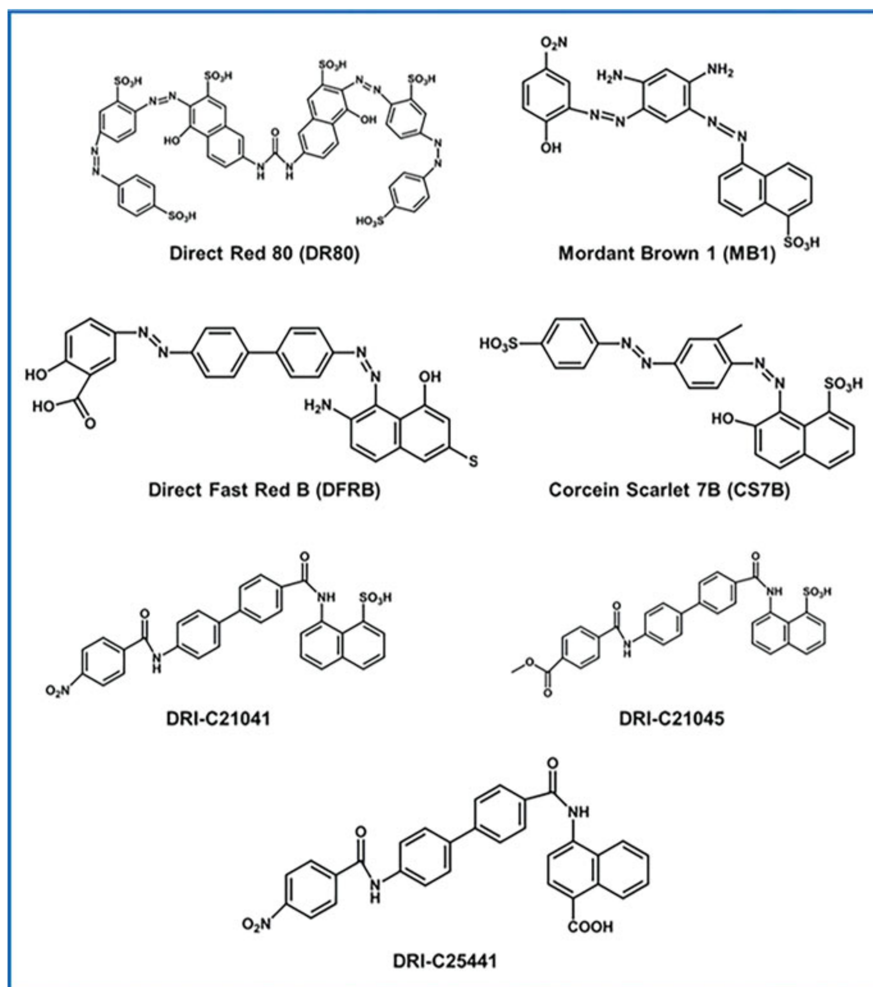


Fig. 7.27 Small-molecule organic dyes investigated as inhibitors against CD40/CD40L interacting protein partners

lying in the low micromolar range. Such organic dyes were more effective in inhibiting the CD40/CD40L interactions in contrast to the interactions related to TNF (tumor necrosis factor) and BAFF (B-cell activating factor) with their cognate receptors. Further, flow cytometry experiments proved the potency of the organic dyes to block the CD40L-induced surface expression of CD40, CD40L, and MHC class II. It has been suggested that these dyes can serve as initial points for drug discovery against CD40/CD40L interactions, and also to understand and investigate the structure-activity relationships involving CD40/CD40L co-stimulatory interactions (Margolles-Clark et al. 2009b). Further, Margolles-Clark reported that mordant brown (a naphthalene sulfonic acid derivative) possesses high activity,

effectivity, and specificity; binding experiments showed that it inhibits the CD40/CD40L interactions at sub-micromolar concentrations, and also exhibits 100 times selectivity for CD40/CD40L interaction contrary to other members of TNF superfamily pairs including OX40/OX40L and RANLK/RANKL (Margolles-Clark et al. 2010).

Chen et al. synthesized a series of compounds on the basis of structures of small-molecule organic dyes previously reported by Margolles-Clark to inhibit the interactions between CD40/CD40L protein pair. Authors reported the IC_{50} of the three compounds including DRI-C25441, DRI-C21045, DRI-C21041 were 0.36, 0.17, and 0.31 μM, respectively (Fig. 7.27). All the reported compounds also displayed inhibitory actions toward various processes including CD40L-induced B-cells activation, their proliferation, and NF-κB activation. Additionally, these compounds have also shown inhibition to the alloantigen-induced immune responses (Chen et al. 2017).

7.10 Inhibitors of Skp2/Skp1 Interactions

UPS (Ubiquitin protein degradation system) is one of the major protein degradation pathways comprising of more than 1000 proteins. UPS being the major pathway participating in various cellular processes that include intracellular signaling pathways, regulation of cell cycle, gene transcription, immune surveillance, etc. Hence, any aberration in the UPS can lead to several diseases (Skaar et al. 2013; Frescas and Pagano 2008). UPS comprises of E1 (ubiquitin-activating enzyme 1), E2 (ubiquitin–conjugating enzyme 2), E3 (ubiquitin-protein ligase 3), and proteasome (Chaugule and Walden 2016). Ubiquitin-protein ligase 3 is one of the most studied class of UPS. One of the most crucial ubiquitin ligases is Skp1-cullin 1-F-box (SCF), a ubiquitin-containing F-box protein. Structurally, SCF comprises of four units including Skp1, Cull, F-box, and Rbx1 (Skaar et al. 2014). E3 ligase is formed as a resultant of association of Skp2 (S phase kinase-associated protein 2) with Skp1, Rbx1, and F-box (Skaar et al. 2014). E3 ligase participates in catalyzing the cellular transformation from G1 to S phase (Hao and Huang 2015). An elevated Skp2 expression has been observed in numerous cancer cell types, which is involved in promoting their invasion and metastasis (Hershko 2008). E3 ligase exerts its functional activity upon the formation of complex between Skp2 and SCF. Skp2/SCF complex formation relies on the interactions pertaining the Skp2/Skp1 protein pairs. This implies that interfering with Skp2/Skp1 interactions averts the complex formation between Skp2 and SCF, which may result in prohibition of appearance and augmentation of tumors (Cai et al. 2020; Morrow 2018).

Zheng et al. solved the crystallographic structure of Skp2/SCF complex and SCF and observed a direct interaction between Skp2 and Skp1 via F-box domain and an indirect interaction occurs between Skp2, Cul1, and Rbx1 (Zheng et al. 2002). Chan et al. marked that large interacting interface of Skp2–Skp1 interaction; several residues have contributed significantly to their binding in contrast to other interacting residue pairs. Such residues were named as hot spots. On the basis of

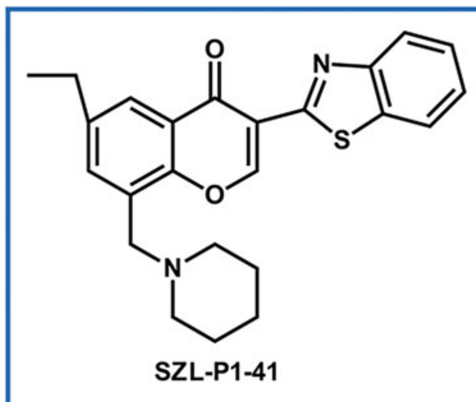


Fig. 7.28 Chemical structure of the most potent inhibitor designed against Skp2/Skp1 interactions

molecular visualization, hot spot analysis, and literature reports, authors found the involvement of 19 hot spot residues for the Skp2/Skp1 interactions. Those 19 residues present on Skp2 have been classified into two pockets like distinctive regions. The initial region represents the pocket 1 that lies in the vicinity of amino-terminus of Skp2 that is located within the F-box motif containing W97, F109, Q116, K119, and W127. The other region which is denoted as pocket 2 lies in the vicinity of C-terminus of Skp2 and constitutes a leucine-rich repeat along with few residues belonging to the F box. Therefore, it is suggested that inhibitors capable of binding these pockets would be effective to impede the Skp2-Skp1 complex formation (Chan et al. 2013).

Chan et al. identified seven small-molecule inhibitors against Skp2/Skp1 interaction. Authors screened 120,000 chemical compounds by employing HiPDock program (Zhang et al. 2008; Zhang and Du-Cuny 2009). Twenty-five compounds showed potency to selectively interact with Skp2. Among them, 16 compounds showed affinity toward pocket 1, nine showed binding affinity toward pocket 2, and two compounds showed binding on both the pockets. Selection of 25 hits was not only based on their binding affinities, but also based on the other drug like features including their size, molecular weight, and solubility. It has been proposed that hits either binding to one of the pockets or both the pockets, may block the interaction of Skp1 and Skp2, thereby restraining the Skp2/Skp1 complex formation. Further, experimental studies including GST pull down assay revealed that seven compounds out of 25 strongly inhibited the formation of Skp2/Skp1 complex. Further, in vitro studies depicted that one of the compounds known as SZL-p1-41 (Fig. 7.28) is the most potent inhibitors of Skp2/Skp1 complex, as it disrupted the interactions even at 5 μM concentration. SZL-p1-41 hampered the formation of Skp2/Skp1 complex in vivo in a dose-dependent manner. Molecular docking studies of complex of SZL-p1-41 with Skp2 demonstrated that SZL-p1-41 interacts with pocket 1 instead of pocket 2 of Skp2, thus revealing the importance of pocket1 during the interactions of Skp2 and Skp1. Docking studies also showed that benzothiazole group of SZL-p1-41 interacts with W97 via a polar contacts and aromatic ring stacking

interactions. Chromone moiety of SZL-p1-41 interacts with D98 and W127 via a hydrophobic, aromatic, and/or hydrogen bonding interactions. Ethyl group present on the phenol ring is not directly involved in the interaction but is located around the position where Skp1 occupies while interacting with Skp2. Hence, this moiety is crucial to impede the Skp2/Skp1 interactions. According to the structure-activity relationship studies, it has been reported that removal of ethyl group moiety indeed abrogates the activity of the inhibitor. Piperidine group of SLZ-p1-41 also plays an imperative role as it is involved in interaction with two important residues including D98 and W127. Further, both in vivo and in vitro experiments demonstrated the prohibition of Skp2-mediated ubiquitination of p27 by Skp2 inhibitors. Further, it has also been proclaimed that SZL-P1-41 is a potential inhibitor for the tumor growth. Additionally, it has also been noted that Skp2 inhibitors not only halt the Skp2/Skp1 complex formation, but also curtailed the ligase activity of Skp2 E3. It has been noted that high dosage of SZL-P1-41 can also significantly affect the expression levels of Skp2 (Chan et al. 2013).

7.11 Inhibitors of Keap1/Nrf2 Interactions

Keap1/Nrf2/ARE signaling pathway is an imperative pathway related to antioxidant stress and deviation of the signaling events that results in numerous diseases like neurodegenerative disorders (such as Parkinson's and Alzheimer's), inflammatory diseases (such as arthritis, diabetes), and cancer (Tkachev et al. 2011). Keap1 targets NRF2 under physiological conditions to commence ubiquitin-based protein degradation. Under the oxidative stress conditions, Nrf2 enters the nucleus by escaping out Keap1-mediated degradation. While present in nucleus, Nrf2 leads to the activation of cytoprotective and antioxidant genes (Padmanabhan et al. 2006; Zhang 2006). Hence, the activators of Nrf2 signaling pathway may serve as therapeutic agents against diseases related to oxidative stress. Most of the Nrf2 activators halt the Keap1/Nrf2 interactions, by interacting with Keap1. Nrf2 activators interact by the formation of covalent bond with sulfhydryl groups present in cysteine of Keap1 via alkylation or oxidation. Activator upon interaction with Keap1 changes its conformation, thereby prohibiting its interaction with Nrf2 (Hong et al. 2005). The covalent binding of activator with Keap1 is irreversible, and results in accumulation of active Nrf2, which has been correlated to other complications like cancer (Zhang 2010). Hence, a novel therapeutic strategy has been proposed involving interference of Keap1–Nrf2 interactions by directly using noncovalent small molecules. Such noncovalent molecules are potent and directly dissociate the Keap1/Nrf2 complex and exert antioxidant defense effects (Magesh et al. 2012).

Lo et al. divulged the PPI interface between Nrf2 and Keap1 by solving the crystallographic structure of a complex obtained by interactions of the Keap1 Kelch domain and a peptide derived from Nrf2. Authors found that Keap1 residues including R380, R415, R483, S363, S508, S555, S602 are the key residues that play crucial role in interactions between Keap1 and Nrf2. These interacting residues are now being utilized to design the inhibitors against the Keap1/Nrf2 interactions

(Lo et al. 2006). Number of peptide-based inhibitors have been designed against Keap1/Nrf2 (Hancock et al. 2012, 2013; Georgakopoulos et al. 2018; Wells 2015). Hu et al. designed several fluorescent probes to establish that the peptide that comprises of nine amino acids possesses an optimum length and activity to interfere with the interactions between Keap1 and Nrf2. Fluorescent probes were used to design a peptide-based inhibitor P1, which showed moderate inhibitory activity with IC_{50} value of 3.5 ± 0.9 μM against Keap1/Nrf2 interaction. The activity upsurge has also been observed with an increase in the length of peptide. Hexadecapeptide (P2) showed highest activity, with an IC_{50} value of 163 nM (Hu et al. 2013). Neutralizing positively charged groups at N-terminus of the peptide via acetylation has significantly changed its electrical characteristics. A nonapeptide P3 also showed high activity with IC_{50} equal to 194 nM. C18 fatty acid stearic heptapeptide displayed exquisite activity ($IC_{50} = 22 \pm 3$ nM) (Hancock et al. 2013).

Owing to the large molecular size of such peptides and their poor penetration ability in cell membrane, efforts were made in the direction to reduce the size of such inhibitory peptides and increase their membrane permeability. Further, Salim et al. designed a macrocyclic peptide inhibitor against interactions between Keap1 and Nrf2. Authors covalently linked a previously reported cyclic peptidyl inhibitor against Keap1/Nrf2 interactions to a cyclic CPP (cell-penetrating peptide). Resultant bicyclic peptide that retained its binding affinity toward Keap1 was resistant to proteolytic cleavage, and permeable to mammalian cells. It also showed an ability to activate transcriptional activity of Nrf2 at low nanomolar concentrations in cell culture. The designed inhibitor presents a potential approach to investigate the functionality of Keap1-Nrf2 associated pathways, and can serve as initial points to develop anticancer and anti-inflammatory agents (Salim et al. 2020). Keeping in view the low-cell permeability of peptides with high-binding affinity for Keap1, researchers across the globe have also designed small-molecule inhibitors against Keap1/Nrf2 interactions (Marcotte et al. 2013; Sun et al. 2014; Bertrand et al. 2015; Zhuang et al. 2014; Jiang et al. 2014, 2015, 2018; Davies et al. 2016; Abed et al. 2020; Lu et al. 2020b).

7.12 Inhibitors of PD1/PD-L1 Interactions

PD1/PD-L1 signal transduction pathway participates in tumor immune evasion and its development (Dermani et al. 2019). PD1, also called CD279, is a member of CD28 superfamily of T-cell regulatory receptors. PD1 is an immunosuppressive receptor, and PD-L1 is its natural ligand. Under normal conditions, the expression of PD1 on activated form of immune cells, endorses T-cell maturation and aids in regulation of the immune response for maintaining immune tolerance. Cancellation of immune system surveillance occurs under negative regulation of T cells which is a resultant of overactivated PD1/PD-L1 signaling pathway. This causes the tumor cells to escape immune response and allows the cells to further develop (Pardoll 2012; Liu et al. 2021; Han et al. 2020). Hence, it has been suggested to be a highly potent approach to interfere the PD-1/PD-L1 interactions, and thereby sustaining the

immune functioning of T-cells in order to treat tumor (Akinleye and Rasool 2019; Liu et al. 2021). Several PD1 and PD-L1 inhibitors including antibodies, peptide-based inhibitors, and SMIs have been engineered (Konieczny et al. 2020; Akinleye and Rasool 2019; Liu et al. 2021).

Monoclonal antibody-based drugs including Bavencio (Avelumab), Opdivo (Nivolumab), Pembrolizumab (Keytruda), Tecentrig (Atezolizumab), Imfinzi (Durvalumab) have already been validated as drugs that hinder the PD1/PD-L1-associated interactions to treat melanoma, NSLC (nonsmall cell lung cancer), and many other diseases (Dirix et al. 2018; Ferris et al. 2016; Garon et al. 2015; Antonia et al. 2017; Socinski et al. 2018). Pembrolizumab, first PD1 inhibitor that attained approval by FDA to treat advanced melanoma (Najjar and Karaman 2019). Pembrolizumab represents a humanized IgG4-κ anti-PD1 monoclonal antibody that exerts its actions by activating the tumor infiltrating lymphocytes (TIL). The association of expression of PD-L1 and PD1 in tumor cells and TILs are the major culprits for tumor immune escape. Pembrolizumab interacts with PD1 and thereby masks its interactions with PD-L1 in order to activate tumor-infiltrating lymphocytes (TILs). Although immune therapeutics against PD1/PD-L1 interactions have gained clinical approval, usage of such monoclonal antibodies (MAbs) exert severe side effects. MAbs have been reported to adversely affect the T-cells' proliferation and activation, resulting in tissue damage and killing of immune cells (Hwang et al. 2016; Naidoo et al. 2015).

Chang et al. employed mirror-image phage display technology and obtained D-peptide antagonist against PD1/PD-L1 signaling pathway. D-Peptide was highly resistant to hydrolysis. Optimization attempts by authors resulted in an optimized compound, (D) PPA-1 that showed in vitro binding to PD-L1 with a binding efficacy of 0.51 μM. Further, cell-based assays and mice experiments marked that (D)-PPA-1 is highly potent in interrupting the interactions pertaining PD1 and PD-L1 under in vivo conditions. Such D-peptide-based antagonists represent a new class of drugs with low-molecular weights for the cancer treatment. The combined efforts of companies Pierre Fabre and Aurigene Discovery Technologies Ltd. (ADTL) led to the emergence of AUNP12. AUNP12 is a 29-mer peptide which acts as immune checkpoint modulator of PD1/PD-L1 signaling cascade. The peptide showed high activity against HEK293 cells expressing hPD-L2. It has also been reported that AUNP12 is capable to interfere with the growth of B16F10 mouse melanoma cells. For instance, under in vitro conditions, AUNP12 blocked the interactions between PD1 and PD-L1 with IC_{50} value 0.72 nM. Animal studies depicted that AUNP-12 possesses high anti-PD-L1 activity and is also highly efficacious in interfering in tumor growth and its metastasis. Structural modifications of AUNP-12 were also carried out to further improve its activity (Sasikumar et al. 2011). Further, macrocyclic peptides (MCPs) were designed against PD1/PD-L1 interactions by BMS (Bristol Myers Squibb) company (Miller et al. 2014b,a). Magiera-Mularz et al. provided insights into the binding modes and interactions pertaining to the formation of complex between MCPs and PD-L1. Authors reported that MCPs can directly interact with PD-L1 and antagonize its functioning which is similar to that of

antibodies. MCPs, thereby aid in restoration of functioning of T cells (Magiera-Mularz et al. 2017).

Lack of molecular details about the structures of PD1 and PDL-1 hindered the advancement of SMIs against PD1/PDL-1 protein–protein interaction. In order to gear up the progress to design SMIs against the interactions between PD1 and PDL-1; Zak et al. unraveled the structural details of complex between PD1 and PD-L1. Authors reported that the interfacial region of PD1 and PD-L1 comprises of three important binding pockets. Revealing molecular level details about the PD1/PD-L1 complex formed the basis to develop small-molecule inhibitors against PD1/PD-L1 signaling pathway (Zak et al. 2015). Bristol Myers Squibb (BMS) performed a comprehensive investigation with respect to SMIs against PD1/PD-L1 interactions. BMS designed small-molecule inhibitors targeting PD1/PD-L1 axis by substituting the biphenyl group and further connecting it to aromatic ring via a benzyl ether bond. BMS employed HTRF (homogenous time-resolved fluorescence) assay coupled with the usage of europium cryptate-labeled anti-Ig in order to figure out the binding affinities of such inhibitors. IC_{50} values for SMIs were lying in the range of 0.6 nM to 20 μM (Chupak et al. 2005; Chupak and Zheng 2014). Further, structural modifications and optimization resulted in compounds with improved binding affinities that were lying in the range of 0.6 nM to 10 nM (Chupak et al. 2005). Researchers were indulged in optimizing the structures of the SMIs against PD1/PD-L1 signaling cascade (Yeung et al. 2016, 2017a, b, 2018).

7.13 Inhibitors Against GTPases

Small GTPases belong to the family of GDP/GTP-binding proteins. They perform their functions as hydrolase enzymes that bind to GTP and convert it to GDP. They act as molecular switches to control the variety of cellular functions related to normal/diseased state (Cromm et al. 2015). Small GTPases exert their regulatory functions by interacting with a variety of other proteins. Small GTPases are active when they are bound to GTP and remain in inactive form when bound to GDP. The cycle between the active and inactive versions of small GTPases depends on three regulatory proteins that include: (1) GEFs (guanine nucleotide exchange factors), (2) GAPs (GTPase-activating proteins), (3) GDIs (guanine nucleotide dissociation inhibitors). When GEFs bind to small GTPases, GDP dissociation and GTP association occur (Cherfils and Chardin 1999). In contrast, GAPs inhibit GTPases by binding to them, and thereby inducing the hydrolysis of GTP to GDP (Tcherkezian and Lamarche-Vane 2007). Hence, they aid in maintaining the appropriate activity levels of GTPases. GDIs are the inhibitors that inhibit the GDP dissociation from GTPases. They hold the inactive form of GTPases in order to circumvent the exchange of GDP to GTP (DerMardirossian and Bokoch 2005).

Small GTPases are classified into five major sub-families including Ras, Rab, Rho, Arf, and Ran on the basis of their structure, sequence, and function. They exhibit affinity toward a variety of effector proteins to regulate the downstream signaling pathways. Ras GTPase interacts with at least 11 distinct downstream

effector families, which in turn activates a variety of genes related to cellular growth, differentiation, and survivability. Among them, P13K-AKT-mTOR and RAF-MEK-ERK pathways have attracted much attention, as any aberration in these pathways is associated to plethora of cancers (Papke and Der 2017). Rho GTPases possess more than 60 downstream effector molecules including WASP (Wiskott-Aldrich syndrome protein) and ROCK (Rho-associated protein kinase), that are involved in regulating the shape of cells, their adhesion, and migratory activities (Lu et al. 2009). Rab GTPases contain 60 members that participate in regulating the intracellular trafficking via an interaction with various effector proteins including Rabenosyn5, and Rabphilin (Hutagalung and Novick 2011). Arf GTPases comprise of around 30 members that are also involved in regulating the intracellular trafficking via effector molecules including binder of ARL2 (BART) and GGA (Golgi-localized gamma-ear-containing ARF-binding) (Khan and Ménétrey 2013). Ran GTPases play crucial role in nuclear processes that include maintenance of the structure of nucleus, import of proteins, mRNA processing, regulation of cell cycle. They perform these functions by interacting with various effector molecules including RanBP1 and RanBP2 (Avis and Clarke 1996).

Small GTPases perform manifold roles in distinct cellular processes by interacting with various protein partners. Hence, it is a promising approach to target the PPIs associated with small GTPases for their effective functioning and development of novel therapeutics for the treatment of related diseases (Gray et al. 2020) Several of the inhibitors targeting PPIs related to GTPases are discussed briefly in the following subsections.

7.13.1 Small Inhibitors Against Ras

GEF-binding site of GTPases is being targeted in order to hinder the interactions pertaining GEF and GTPases. This will block the formation of complex between GEF and GTPase, and the GDP turnover. Small molecules and peptides have been designed that either interfere with the interactions between GEF and Ras or they are capable of binding to Ras, thereby, locking its conformational state which is not favorable for binding to GEF (Gray et al. 2020).

Taveras et al. designed SCH-54292 inhibitor (Fig. 7.29) that deactivates Ras by binding in a competitive fashion with GDP. NMR studies revealed that SCH-54292 and the related compounds interact with major hydrophobic cleft located in switch II region of RAS (Taveras et al. 1997). It was found that such compounds cannot be developed as probes/drugs owing to their poor stability and low solubility in organic or aqueous solvents. These initial hits lead to the development of novel compounds by adding linking spacer between the two aromatic pharmacophores that resulted in the improvement in its aqueous solubility. Series of compounds with arabinose-derived bicyclic linker were proved to be potent with mild cytotoxic effects on oncogenic Ras-expressing cells (Tisi et al. 2020). It has been proposed that this series of molecules hinder the nucleotide exchange by interfering with the binding of GEF to Ras GTPase (Lee et al. 2020). Sun et al. screened 11,000 fragments using NMR

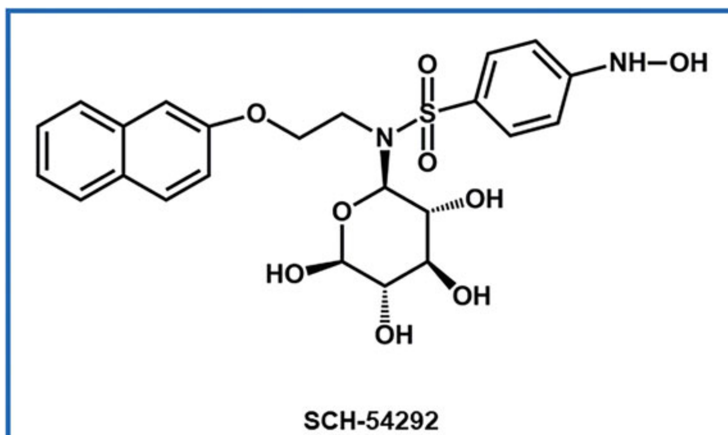


Fig. 7.29 Chemical structure of SCH-54292 inhibitor designed to deactivate Ras

and obtained 140 hits that showed binding affinity ranging from 1.3 to 2 mM. X-ray crystal structure of 20 compounds complexed with KRAS showed that the compounds bind in the hydrophobic pocket located between the switch II helix and central β-sheet of the protein. Based on the structural analysis of these compounds, a number of indole analogs were synthesized and compounds with improved binding affinity were obtained. It has been suggested that such compounds can be employed as probes for the development of covalent "tethering" compounds to interact with the binding site and saturate it. This provides an opportunity to screen fragments for the second binding pocket. Fragments obtained from the second screening showed affinity in the millimolar range, which can be improved to micro- or nanomolar range by further chemical optimization (Kessler et al. 2019).

7.13.2 Peptide-Based Ras Inhibitors

Peptidomimetics have been designed to block the interactions between RAS and SOS (Ras-specific GEF). SOS interacts with Ras via its helical hairpin comprising of two helices (αH and αI helices) as such, that the helices interact with switch regions contained in Ras. αH helix of SOS is involved in direct contact with Ras; hence, mimics of αH helix of SOS have been designed to interfere with the interactions between RAS and SOS. Further structural and mutational studies depicted the crucial roles of F929 and N944 of αH in binding to Ras. This augmented the design of helical mimetics of full length (929–944) αH helix of SOS. Authors employed hydrogen bond surrogate strategy to design αH helical mimetics. Using HBS (Hydrogen bond surrogate) strategy, authors have replaced the main chain hydrogen bond at the N-terminus, bonding between the carbonyl group of *i*th and the amine group of $i + 4$th residue by the covalent C–C bond. It has been marked that helices obtained via HBS approach usually possess high-binding affinity and specificity

toward their target (Henchey et al. 2010a, b). Synthetic mimics were further optimized to enhance their solubility by the incorporation of charged residues at the noninterfacial positions. Optimized HBS helix showed potent inhibitory effect against the interactions between Ras and SOS (Patgiri et al. 2011).

Leshchiner et al. have also designed numerous stapled helical peptides on the basis of structure of αH helix of SOS that spans from residues 929 to 944. Stapled peptides were screened by fluorescence polarization assay for their binding avidity to KRas and its mutant forms including G12V, G12D, G12S, G12C, and Q61H. Authors found lead peptides named as SAH-SOS$1_{a,c,d}$ that showed their binding affinity lying in the nanomolar range (60–160 nM). Authors further revised the sequence of SAH-SOS1_a peptide by incorporating two Arg residues at the N-terminus in order to fine tune the charge of the peptide, which enhanced its solubility and cell penetration ability. Further biochemical and cell-based studies confirmed the potency of SAH-SOS1_a to interfere with the interactions between KRas and SOS (Leshchiner et al. 2015).

7.13.3 Inhibitors for RasGEF (SOS1)

Evelyn et al. designed an inhibitor that binds with SOS1, and thereby prevents the interaction between SOS1 and Ras. Authors identified a lead inhibitor named NSC-658497 (Fig. 7.30a), via a rational designing approach that involved virtual screening coupled with experimental screening and validation. Further, mutational studies and structure-activity relationship studies deciphered the functional moieties of NSC-658497 that are involved in interaction with SOS1 catalytic site. This small-molecule inhibitor showed dose-dependent efficacy to block Ras-associated down-stream signaling pathways. It has been marked that such a highly effective synthetic inhibitor can be subjected to further optimization protocols for their development as future drugs (Evelyn et al. 2014).

Hilling has also identified a series of effective SMIs with an ability to block the interactions of Ras with SOS1. Authors showed that these inhibitors lead to antiproliferative effects by preventing the formation of complex between KRAS

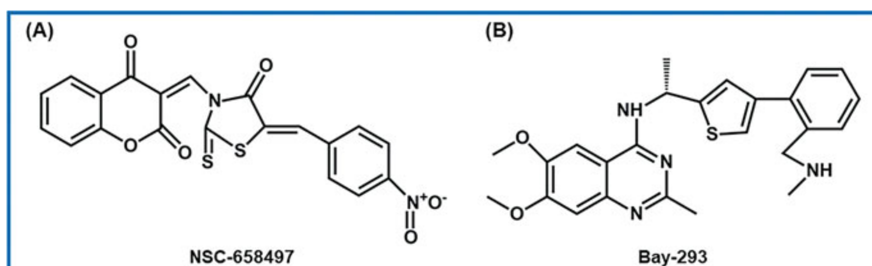


Fig. 7.30 Chemical structures of SMIs designed against the interactions between SOS1 and (**a**) RAS and (**b**) KRAS

and SOS1 to ultimately block the reloading of KRAS with GTP. Lead compound named as Bay 293 (Fig. 7.30b) showed IC_{50} value of 21 nM to disrupt the interactions between KRAS and SOS1 (Hillig et al. 2019). Several other inhibitors acting as the blockade for the regulatory actions of RasGEF have also been fabricated and have been summarized recently by Gray et al. (2020). Several companies are dedicated toward the designing of inhibitors against KRAS and some of the promising inhibitors identified have reached phase I/II clinical trials (Gray et al. 2020).

7.14 Conclusion

This chapter highlights the various inhibitors targeting protein–protein interactions involved in severe diseases such as cancer, infections, and hereditary disease. A series of biological, biophysical, and computational methods have substantially influenced the study of PPI inhibitors over the years. Whilst the strategy of developing PPI-targeted inhibitors is rather recent, the usage of inhibitors having potency to inhibit such interactions has been known since the age of penicillin. However, the translation of such drugs in clinical setup is a great challenge owing to the lack of efforts in comprehensive data acquisition, validation, and interpretation. Despite of such obstacles, the ease of scalability, and structural malleability, the development of small-molecule inhibitors is promising in the field of drug discovery and therapeutics.

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