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PDZ Domains as Drug Targets

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Protein–protein interactions within protein networks shape the human interactome, which often is promoted by specialized protein interaction modules, such as the postsynaptic density-95 (PSD-95), discs-large, zona occludens 1 (ZO-1) (PDZ) domains. PDZ domains play a role in several cellular functions, from cell–cell communication and polarization, to regulation of protein transport and protein metabolism. PDZ domain proteins are also crucial in the formation and stability of protein complexes, establishing an important bridge between extracellular stimuli detected by transmembrane receptors and intracellular responses. PDZ domains have been suggested as promising drug targets in several diseases, ranging from neurological and oncological disorders to viral infections. In this review, the authors describe structural and genetic aspects of PDZ-containing proteins and discuss the current status of the development of small-molecule and peptide modulators of PDZ domains. An overview of potential new therapeutic interventions in PDZ-mediated protein networks is also provided.

1. Introduction

In the post-genomic era, it has become clear that it is not the number of genes that determines biological function and complexity, but rather the intricacy of interactions between proteins, also known as the interactome,[1] where more than 600 000 unique interactions have been described in humans.[2] Several recent, large-scale efforts have attempted to describe these protein networks, often using a combination of pull-down assays and mass spectrometry analysis, thereby describing thousands of protein–protein interactions (PPIs), thus approaching a complete map of the human interactome.[3–7] Hence, our knowledge of the human interactome is improving dramatically, while it has been known for decades that PPIs are vital for almost all cellular and biochemical processes and are attractive as novel and promising drug targets.[8–10]

Interestingly, a vast number of PPIs are mediated by protein interaction modules or domains that have evolved to recognize specific elements of partnering proteins. Often, these protein domains are found in several different proteins of the human proteome. In this review, we focus on the postsynaptic density-95 (PSD-95), discs-large, zona occludens 1 (ZO-1) (PDZ) protein domain family, which is one of the largest classes of PPI domains in the human proteome. PDZ domains typically increase the specificity and efficiency of intracellular communication networks downstream of receptor activation by facilitating several PPIs.[11] PDZ domains are often found in multidomain scaffold and anchoring proteins involved in trafficking, recruiting, and assembling of intracellular enzymes and membrane receptors into signal-transduction complexes.[12,13] Many PDZ domains are emerging as drug targets,[14–16] and in addition to their physiological and therapeutic relevance, PDZ domains are frequently used as a model system to explore fundamental aspects of protein folding,[17] ligand–protein interactions,[16,18] and allostery.[18,19]

2. PDZ Domain Structure and Function

Since the discovery of PDZ domains in the early 1990s,[20–22] a total of 268 PDZ domains have been identified in 151 distinct human proteins. PDZ domains are small modular protein entities consisting of 80–110 residues. Despite a very low sequence similarity, where few regions are conserved (**Figure 1** and Figure S1, Supporting Information), PDZ domains share a compact globular fold generally comprising five to six antiparallel β-strands and two α-helices in a βA-βB-βC-αA-βD-βE-αB-βF arrangement (**Figure 2**a). However, a few PDZ domains such as those of Golgi reassembly stacking protein 1 (GORASP1) and GORASP2 deviate from this common secondary structural arrangement by comprising a βC-αA-βD-βE-αB-βF-βA-βB

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configuration while maintaining the overall globular fold of PDZ domains (Figure 2b).[23] The N- and C-terminal regions of PDZ domains are often in close proximity, which may have facilitated an evolutionary insertion of PDZ domains into larger proteins.[24] These structural characteristics were first recognized in 1996 by the structural determination of the third PDZ domain of disks large homolog 1 (DLG1), also known as synapse-associated protein 97 (SAP-97)[25] and has since been supported by X-ray crystal and NMR structures for more than 150 different human PDZ domains, representing approximately 100 different proteins.[26]

PDZ domains were initially recognized by their ability to bind consensus motifs in the C-terminal extremity of their protein interaction partners. The affinity toward such carboxy-terminal peptide sequences has been predicted to be dictated primarily by the very C-terminal amino acid (referred to as P_0) and the residue at position −2 (P_{-2}) of the peptide ligand (Figure 2d). This led to the classification of PDZ domains into three main classes:[24,27] Class I PDZ domains recognize C-terminal peptides with T/S-X-Ψ (with T and S being Thr and Ser, respectively, X being any amino acid, and Ψ being a hydrophobic residue) motifs, whereas class II and class III PDZ domains preferentially bind Ψ-X-Ψ motifs and D/E-X-Ψ motifs (with D being Asp and E being Glu), respectively. In this binding mode, known as the canonical binding mode, the peptide carboxylate arranges as an antiparallel β-strand in a hydrophobic cleft between βB and αA of the PDZ domain, where the C-terminal carboxylate binds to a loop between βA and βB through a network of hydrogen bonds (Figure 2a,d). Subtle backbone amide-to-ester substitutions, in either the PDZ domain or ligand, can disrupt the hydrogen bonding network and lead to a substantial reduction in binding affinity.[28,29] The carboxylate binding site has generally been identified as a highly conserved Gly-Leu-Gly-Phe motif, also known as the GLGF loop. However, an alignment of primary sequence and secondary structural features of PDZ domains demonstrate greater variability in this region and suggest a more generalized Ψ-Gly-Ψ carboxylate binding motif, where only the second Gly is conserved among most human PDZ domains (Figure 1 and Figure S1, Supporting Information). This Gly residue adopts a left-handed α-helical conformation creating the loop required for coordinating to the carboxylate of the peptide ligand.[30] The hydrophobic cavity created by the Ψ-Gly-Ψ loop varies in size among the human PDZ domains, allowing various hydrophobic side chains in P_0 and thereby contributing to the selectivity.[27] In class I PDZ domains, the Thr or Ser in P_{-2} of the ligand forms a hydrogen bond with the imidazole side chain of a highly conserved His residue in the N-terminus of αB (Figure 2a). Other PDZ domains have a conserved Tyr in the corresponding position, which possesses greater electrophilicity than His, and may explain the preference for acidic amino acids in P_{-2} of some class III PDZ domains.[31]

The early simplistic classification of PDZ domains into three classes is still debated; later studies have revealed that PDZ domains are generally highly promiscuous PPI modules,[32] and that the mechanism of PDZ domain binding is more complex than initially believed.[33,34] In a number of PDZ domain structures, the peptide ligands bind perpendicular to the PDZ domain in a noncanonical fashion, where only P_0 interacts with the PDZ domain.[35–37] Several other examples are known where, in addition to P_0 and P_{-2}, upstream residues as far as P_{-7} and P_{-14} influence the binding of C-terminal peptide ligands to PDZ domains.[27,38,39] This includes the synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP), where an N-terminal extension (P_{-6} to P_{-14}) is required for the high-affinity interaction with the PDZ domain of synap-

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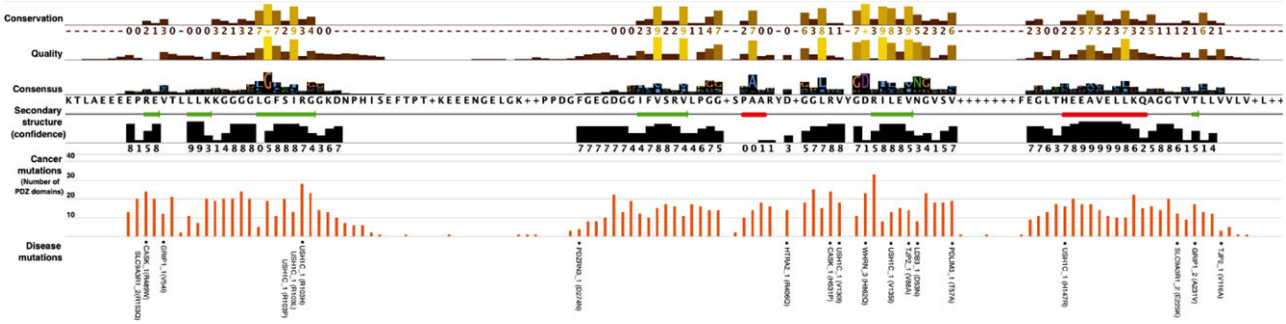


Figure 1. The summary of the sequence alignment of all human PDZ domains. Conservation and alignment quality measures, the best estimate of the sequence consensus, secondary structure prediction (Jpred), numbers of cancer-causing mutations, and the disease-causing mutations. Alignment characteristics calculated and visualized using Jalview software.

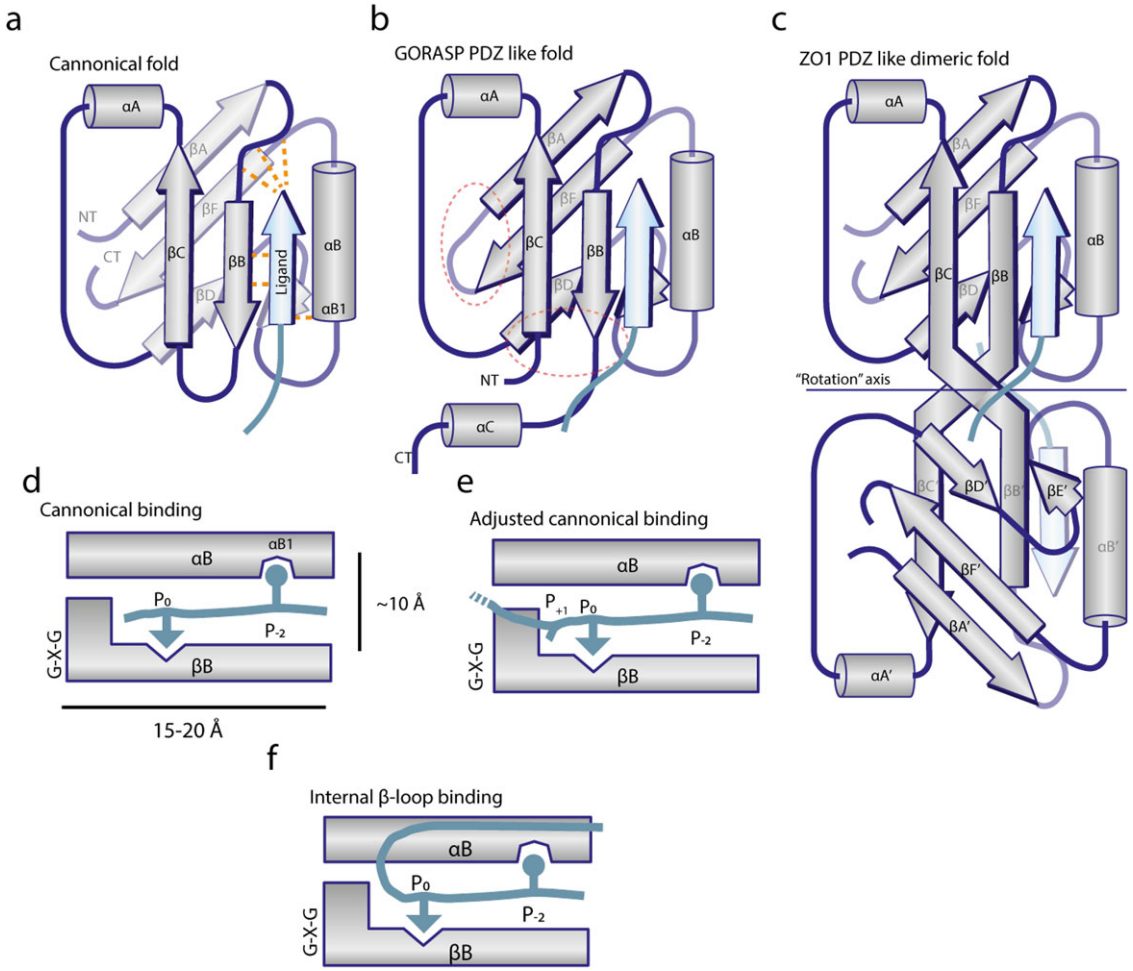


Figure 2. a) Canonical fold of a PDZ domain shows six β-sheets and two α-helices, with a ligand (blue) inserting into a binding pocket between αB and βB. In a canonical insertion, there is a hydrogen bonding network (orange dashed lines) between the backbone of the PDZ domain and the backbone of the peptide. b) In the noncanonical fold of the GORASP1 and two PDZ domain, the overall globular fold remains intact but with N-terminal and C-terminal placed βC and βB, instead of βA and βF, respectively (red dotted spheres). c) In ZO-1 PDZ2, the domain folds from two nonfunctional monomeric domains where βB and βC of the two domains swap in order to complete the fold into the functional dimeric PDZ tandem. d) The canonical insertion mode often relies on interactions between the ligands P_0 carboxylic acid and amine to the backbone of the PDZ domain, and insertion of the P_0 side chain into a hydrophobic pocket; furthermore, the interaction is often strengthened by either insertion or interaction of P_{-2} with a hydrophobic cavity (class II ligand) or a conserved charged residue (class I ligand) at αB1 (often a His). e) PDZ domain ligands can also bind using internal motifs where it usually still relies on the insertion of a hydrophobic residue into the binding pocket and either hydrophobic or hydrogen bond interaction coordination of the P_{-2}, but the interaction also relies on the P_{+1} or P_{+2} residue being negatively charged (Glu/Asp), using the carboxylic acid as substitute for the C-terminal. f) The β-loop binding mode in many ways resembles (e) but the bend of the β-loop allows the ligand to cover twice the surface area and stabilize the β-strand formation of the inserted part through intermolecular hydrogen bonding, overall stabilizing the interaction.

tic scaffolding protein Shank3. In this case, SAPAP docks into a second hydrophobic pocket formed by an N-terminal extension and the βB-βC loop of the Shank3 PDZ domain.[40] In yet further distinctive binding mode variations of the canonical interaction, P_0 is buried deeper in the binding groove of the PDZ domain, occupying what corresponds to the P_1 position (Figure 2e).[41] In an additional case, a Gly at P_{-2} induces conformational flexibility within the ligand backbone, whereby the P_{-3} amino acids shifts to occupy the P_{-2} position.[42] In another example, a Cys in P_0 enables the ligand to bind in two distinct positions, canonical and upward shifted, inserting the P_{-2} amino acid into the conventional hydrophobic binding pocket, and allowing P_{-4} to make a salt bridge to a Lys positioned at the N-terminal of αB, which stabilizes the binding.[38] Some PDZ domains can also bind phosphopeptides,[43] which has been suggested to be involved in phosphoregulation of PDZ domain interactions, thus adding to the plasticity of PDZ domain-mediated interactions.[44,45]

In addition to the carboxylate-dependent binding of PDZ domains, it has more recently been demonstrated that PDZ domains can also bind internal peptide motifs[42,46–49] and phospholipids.[50–52] The interaction between the neuronal nitric oxide synthetase (nNOS) β-hairpin structure and the syntrophin PDZ domain is an example of binding of internal peptide motifs. Here, the entire β-hairpin peptide (Figure 2f), comprising two β-strands connected by a sharp β-turn, is required for

binding, thereby occupying more than twice the surface area of peptides binding to PDZ domains in the canonical carboxylate-dependent fashion. The carboxylate binding loop of the PDZ domain plays an essential role by coordinating to the backbone carbonyl of a Phe at P_0. This Phe has an analogous role to the hydrophobic P_0 residue of canonical C-terminal peptide ligands by occupying the hydrophobic pocket.[49] In the case of phospholipids, PDZ domains generally lack well-defined binding pockets and PDZ-phospholipid interactions have been mapped to different structural regions in distinct cases, as reviewed in detail previously.[44,53] Generally, the negatively charged phospholipid head group is stabilized by positively charged clusters in the phospholipid binding site. Both for syntenin and protein interacting with C Kinase 1 (PICK1), the PDZ-phospholipid interactions have been shown to be crucial for clustering membrane receptors to the cell membrane and,[54–56] for PICK1, in a redox-dependent manner.[56]

In addition to the multifaceted behavior of PDZ domains, several have been shown to form dimeric structures through homo- or heterodimerization.[57–63] For instance, the second PDZ domain of ZO-1 was shown to homodimerize via domain swapping, where βA and βB of one domain is swapped with βA' and βB' from the second domain (domain 1: βA-βB-βC'-αA'-βD'-βE'-αB'-βF'; domain 2: βA'-βB'-βC-αA-βD-βE-αB-βF), which completes the correct fold and shape of the ligand binding pocket (see Figure 2c). For PICK1, Cys residues in the βB-βC loop have been shown to facilitate a redox-dependent homodimerization of the single PDZ domain of PICK1, and it is believed to contribute to the ligand binding capabilities of PICK1.[56]

In the past decade, it has become evident that the structural and functional properties of PDZ domains can be affected by extensions beyond the conventional PDZ domain structural core by forming functional independent units or supramodules.[64] Such extensions can be small secondary structural elements that add to the PDZ domain structural core, as exemplified by the third PDZ domain, PDZ3, of PSD-95 that contains an additional C-terminal α-helix which influences ligand binding through an allosteric mechanism.[65] In other cases, PDZ supramodules are formed by neighboring domains in either a homotypical[36,66–70] or heterotypical fashion (Figure S2, Supporting Information). In the homotypical PDZ1-2 tandem of PSD-95, these two domains are restricted by a short linker, positioning the two domains in an antiparallel fashion. In other proteins, such as Whirlin, a longer and more flexible linker allows for greater flexibility of the tandem PDZ domain.[67] In general, the homotypical PDZ tandem domains can facilitate either binding of multiple copies of single proteins or multiple subunits of protein complexes (Figure S2, Supporting Information), such as the PSD-95 GluN2B interaction. Alternatively, the PDZ tandem domain can bind to different participants in a protein complex, such as one GluN2B subunit and one nNOS binding to one PDZ12 tandem of PSD-95.

However, in some tandem supramodules, the function of one PDZ domain is exclusively to stabilize the other PDZ domain. This is the case for both the PDZ1-2[71] and PDZ4-5[66] tandems of glutamate receptor-interacting protein (GRIP), which comprises seven PDZ domains, and where the folding and binding of one PDZ domain requires the presence of the other PDZ domain. For the PDZ4-5 tandem, PDZ4 exclusively acts as a chaperone for PDZ5 and does not contribute to receptor binding itself.[66,72]

In the heterotypical supramodules, PDZ domains are combined with other modular protein domains including SH3 and guanylate kinase (GK) domains, as seen in the membrane-associated guanylate kinase (MAGUK) superfamily of proteins (Figure S3, Supporting Information). For example, it was demonstrated for ZO-1 that the three domains, PDZ3-SH3-GK, fold into a heterotypical supramodule, increasing the peptide affinity for PDZ3 significantly, compared to the isolated PDZ3 domain.[73] Similarly, for the interaction of MAGUK p55 subfamily member 5 (MPP5/PALS1) with protein crumbs homolog 1 (Crb), the affinity increased 85-fold upon binding to the entire supramodule compared to the isolated PDZ domain.[74] In the case of synaptic Ras GTPase-activating protein (SynGAP) binding to PSD proteins, structural studies show that the PDZ3-SH3 linker region folds into an α-helix upon binding to SynGAP, which stabilizes the overall complex and increases the affinity ≈eightfold and ≈fourfold for PSD-95 and PSD-93, respectively.[75] Hence, the supramodular structures of PDZ domains often fine-tune the specificity and selectivity and should be considered when developing PDZ domain directed inhibitors.

3. Biological Role and Disease Relevance of PDZ Domain Proteins

PDZ domain-containing proteins are highly involved in distinct biological processes, such as cell cycle, signal transduction, and metabolism, according to the available annotations of 74 of the 151 human PDZ-containing proteins in the Reactome database of manually curated, experimentally proven, and peer-reviewed pathways (**Table 1**).[76] While most of these PDZ domain proteins are primarily involved in one pathway group, such as a specific signal transduction or cell cycle, some of them are involved in a plethora of different processes (Table S2, Supporting Information). For example, the 26S proteasome non-ATPase regulatory subunit 9 (PSMD9) occurs in several protein degradation processes throughout the human interactome. Another example is the PDZ domain-containing protein 11 (PDZD11), which participates in transport of small molecules within both vitamin metabolism and microbial host–pathogen interaction pathways. Most pathways include several different PDZ-containing proteins (Table 1 and Table S2, Supporting Information); 64 of the 74 annotated proteins participate in several pathways, recapitulating the most common function of PDZ domain proteins as scaffolding proteins.

In the STRING interaction database,[77] 149 PDZ-containing proteins are listed with experimentally validated or predicted interactions and, of these, 63 form a dense interaction network when mapped using the basic STRING algorithm (**Figure 3**).[78] Though some of the 63 mapped interactions are not mediated by the PDZ domain in the protein, the majority are mediated either through a canonical or noncanonical PDZ binding mode. Some of the PDZ-containing proteins in the network contain more than one PDZ domain and/or a C-terminal PDZ binding motif and several share the same ligands and interactions (Figure 3). This rich cross interaction combined with the pathway data and the

Table 1. Summary of Reactome pathway participation of PDZ-containing proteins.

Reactome pathway group	Number of proteins[a)]	The most enriched protein(s)[b)]	Number of pathways[c)]
Cell cycle	3	PSMD9	13
Cell–cell communication	8	–	1
Cellular responses to external stimuli	1	PSMD9	1
DNA replication	1	PSMD9	3
Developmental biology	12	LIMK1	3
Disease: diseases of signal transduction	4	CNKSR1, CNKSR2	5
Disease: disorders of transmembrane transporters	1	PSMD9	1
Disease: infectious disease	2	PSMD9	2
Extracellular matrix organization	2	–	1
Gene expression (transcription): RNA polymerase II transcription	2	PSMD9	3
Hemostasis	2	–	1
Immune system: adaptive immune system	5	PSMD9	5
Immune system: cytokine signaling in immune system	4	PSMD9	3
Immune system: innate immune system	9	PSMD9	3
Metabolism of RNA	1	PSMD9	1
Metabolism of proteins: post-translational protein modification	3	PSMD9	3
Metabolism: metabolism of lipids	2	–	1
Metabolism: metabolism of polyamines	1	PSMD9	1
Metabolism: metabolism of water-soluble vitamins and cofactors	1	PDZD11	2
Muscle contraction: ion homeostasis	1	NOS1	1
Neuronal system: neurexins and neuroligins	15	–	1
Neuronal system: neurotransmitter receptors and postsynaptic signal transmission	6	DLG4	5
Neuronal system: neurotransmitter release cycle	6	RIMS1	6
Neuronal system: synaptic adhesion-like molecules	3	–	1
Programmed cell death: apoptosis	3	–	1
Signal transduction: death receptor signalling	5	–	1
Signal transduction: GPCR downstream signalling	7	–	1
Signal transduction: intracellular signaling by second messengers	2	–	1
Signal transduction: MAPK family signaling cascades	5	PSMD9	2
Signal transduction: signaling by Hedgehog	2	PSMD9	5
Signal transduction: signaling by Hippo	3	–	1
Signal transduction: signaling by receptor tyrosine kinases	3	ERBIN	2
Signal transduction: signaling by Rho GTPases	18	LIMK1	2
Signal transduction: signaling by WNT	6	DVL2	8
Signal transduction: TGF-β receptor signaling in EMT (epithelial to mesenchymal transition)	2	–	1

[a)] Total number of PDZ-containing proteins participating in particular pathway group; [b)] PDZ-containing protein known to participate in most of the pathways in particular pathway group; [c)] The number of pathways in the pathway group the protein is involved in.

high-network density observed altogether underlines the fundamental importance of PDZ domain interactions.

Since PDZ domain-containing proteins are involved in numerous signaling pathways, it is not surprising that they are also associated with a range of diseases and disorders. Besides the more well characterized involvement of PDZ domain proteins in neurodegenerative and mental disorders, they are also involved in other diseases such as hearing loss and vision disorders, metabolic disorders, kidney and heart conditions (Table S3, Supporting Information).

To provide an overview of, and insight into, the role of disease-causing mutations in proteins containing PDZ domains, an extensive search was conducted using publicly available gene–disease associations for genes encoding human PDZ-containing proteins (Table S3, Supporting Information). These proteins were subsequently examined for disease-causing mutations located in the PDZ domains, as outlined in Table S3, Supporting Information and below.[79–86]

Amino acid variations within PDZ domains are known to be associated with disease in humans. In the PDZ domain of peripheral plasma membrane protein, calcium/calmodulin-dependent serine protein kinase (CASK), frame-shift, stop codon mutations, and His-531-Pro substitution have been demonstrated to be present in cases of mental retardation and

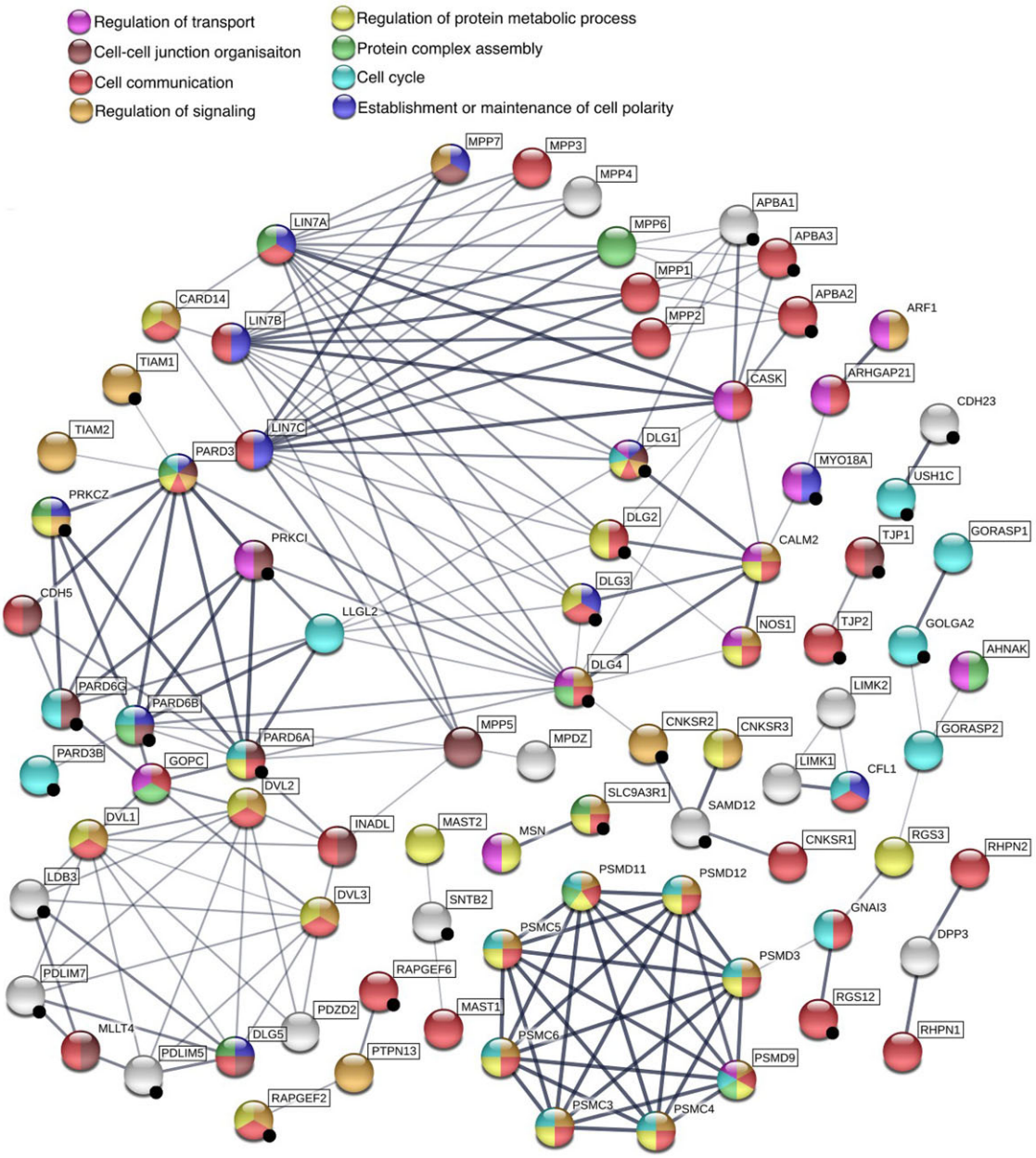


Figure 3. A network of 63 PDZ-containing proteins (names in rectangles) and 20 of their interaction partners (names not marked). Proteins marked with circles are colored according to biological process GO term enrichment. Proteins containing canonical PDZ binding motif in their C-terminus are marked with a black dot. Lines between proteins represent experimental evidence of the protein–protein interaction—the thicker the line, the higher the confidence of the evidence. The figure is made using STRING database information, analysis, and visualization tools.[78]

an Arg-489-Trp mutation in Smith–Magenis syndrome-like developmental disorder (Table S3, Supporting Information). His531 and Arg489 are not adjacent when assessing the protein sequence (Figure 1), but they are in close proximity in the 3D protein structure; moreover, they appear to interact in crystallization experiments.[87] Thus, it can be speculated that mutations in this part of the PDZ domain could disrupt or cause an interaction driving these diseases. A Val-54-Ile mutation in the first PDZ domain, PDZ1, of glutamate receptor-interacting protein 1 (GRIP1), located two residues downstream from a residue with a disease-causing mutation in CASK according to sequence alignment (Figure 1), contributes to abnormalities in brain morphology. In addition, an Ala-231-Val mutation in the N-terminal region of the PDZ2 domain of GRIP1 causes cryptophthalmos syndrome, a skin anomaly causing partial or complete fusion of eyelids.

A mutation in E3 ubiquitin-protein ligase PDZRN3 (Asp-274-Asn) has been shown correlation with schizophrenia. This

mutation is located in a loop known to be involved in interactions with canonical ligands such as the PDZ binding motif of muscle skeletal receptor tyrosine-protein kinase (MUSK).[88]

A number of different mutations in the two proteins harmonin (USH1C) and whirlin (WHRN) are associated with hearing and vision loss disorders, including Usher syndrome (Table S3, Supporting Information). Some of these mutations are located in close proximity to the canonical binding site of the PDZ domain, likely directly interfering with the functionality of the PDZ domains. Numerous disease-causing mutations in PDZ domains are located away from the canonical binding site (Figure 1) and may have an indirect effect on the PDZ domain functionality, such as allosteric effects, perturbation of the 3D structure, modulating multiprotein complex formation, or a combination of these. Examples of these are the mutations in the PDZ domain of HTRA2 which cause metabolic diseases such as 3-methylglutaconic aciduria, mutations in PDZ1 of tight junction protein ZO-2 (TJP2) which cause hypercholanemia. Further examples are the PDZ domains of LIM domain binding protein 3 (LDB) and PDZ and LIM domain protein 3 (PDLIM3) which are related to heart muscle disease cardiomyopathy.

Moreover, mutations in PDZ2 of Na^+/H^+ exchange regulatory cofactor NHE-RF1 (SLC9A3R1) is related to kidney stones. Thus, these, as well as other disease-associated mutation cases (Figure 1), again highlight the role of PDZ domains in several diseases.

4. Pharmacological Targeting of PDZ Domains

Genome-wide association studies have elucidated the disease relevance of mutations in genes encoding proteins containing PDZ domains. However, correction of mutations using gene therapy is currently very limited and not yet available for PDZ domain proteins.[89] Therefore, the modification of a disease pathway is generally achieved by small molecules or peptides that modulate PPIs and selectively interfere with the downstream signaling cascades.

Targeting PPIs has been considered challenging mainly due to their extensive binding interface.[8] This view is now changing since several PPI modulators, binding with high affinity, have been approved as medicines.[9] The majority of PPI modulators interact directly with the surface of one protein target, acting as a competitive antagonist and preventing the formation of the heterodimer product.[90] This strategy has been successfully used to design high-affinity PDZ inhibitors toward neurological and also cancer targets, and one of these ligands has reached late-stage clinical trials.[91] Another approach is based on the allosteric modulation of a PPIs, that is, binding to a protein region that is not within the surface binding site of the two protein partners.[92] Even though allosteric modulation has been used for other protein domains, studies targeting PDZ domains with therapeutic applications are scarce.[93] Finally, two strategies for PPI modulation were recently described but have not yet been applied toward PDZ domain proteins. In the first, instead of inhibiting PPIs using a competitive antagonist, the interaction is stabilized upon ligand binding.[94] In the second, the signaling pathway is modified by a direct reduction of one of the interacting proteins using proteolysis targeting chimera (PROTAC).[95] In the next sections, the applications of PDZ targeting in the treatment of neurological diseases and cancer treatment will be discussed, focusing on the development of orthosteric inhibitors.

4.1. PDZ Domains as Drug Targets in Neurological Disorders

4.1.1. PDZ Domain Proteins Associated with Neurological Disorders

A large number of PDZ domain-containing proteins are associated with neurological disorders. Among others, regulating synaptic membrane exocytosis protein 1 (RIMS1), partitioning defective 3 homolog B (PARD3B), peripheral plasma membrane protein CASK, and disks large homolog 4 (DLG4, PSD-95) are associated with neurodevelopmental disorders, which are central nervous system development disorders with different manifestations.[96] These proteins, like many other PDZ domain proteins, participate in pathways crucial for brain function, such as developmental processes and synaptic regulation (**Figure 4** and Table S2, Supporting Information). PSD-95 and additional PDZ domain-containing proteins participating in nervous system development—gamma-2-syntrophin (SNTG2) and SRC Homology 3 Domain and multiple ankyrin repeat domains proteins 2 and 3 (SHANK2 and SHANK3)—have been linked to autism spectrum disorders, most likely by altering synaptic pathways.[97]

Synaptic PDZ-containing proteins are involved in other neurological disorders, including neurodegenerative diseases. Membrane-associated guanylate kinase, WW and PDZ-containing protein 2 (MAGI2) and MAGUK p55 subfamily member 7 (MPP7), both of which participate in regulation of signaling events, are associated with Alzheimer's disease (AD) (Table S3, Supporting Information). A hallmark of AD is an abnormal aggregation of proteins at synapses,[98] and several synaptic PDZ-containing proteins might be involved in this pathological event. PDZ-containing proteins such as connector enhancer of kinase suppressor of ras3 (CNKSR3), serine protease HTRA2, MAGUK p55 subfamily member 2 (MPP2), nitric oxide synthase (NOS1), and partitioning defective 3 homolog (PARD3) have been linked to Parkinson's disease.[99] Several PDZ-containing proteins are involved in psychiatric disorders and at least eight different PDZ-containing proteins are associated with depression and more than 20 are associated with schizophrenia (Table S3, Supporting Information).

PDZ domains are also associated with hearing and vision disorders (Table S3, Supporting Information). PDZ domain-containing protein GIPC3, tight junction protein ZO-2 (TJP2), harmonin (USH1C), and whirlin (WHRN) are associated with hearing loss. Two of these, USH1C and WHRN, together with two other PDZ-containing proteins PDZD7 and RIMS1, are also associated with retinitis pigmentosa, a genetic disorder affecting sight. There is evidence to suggest that USH1C, WHRN, and PDZD7 are involved in Usher syndrome, a disease affecting both hearing and vision. These are examples of the most well annotated protein–disease relationships; however, additional PDZ-containing proteins are likely implicated in similar pathogenic processes.

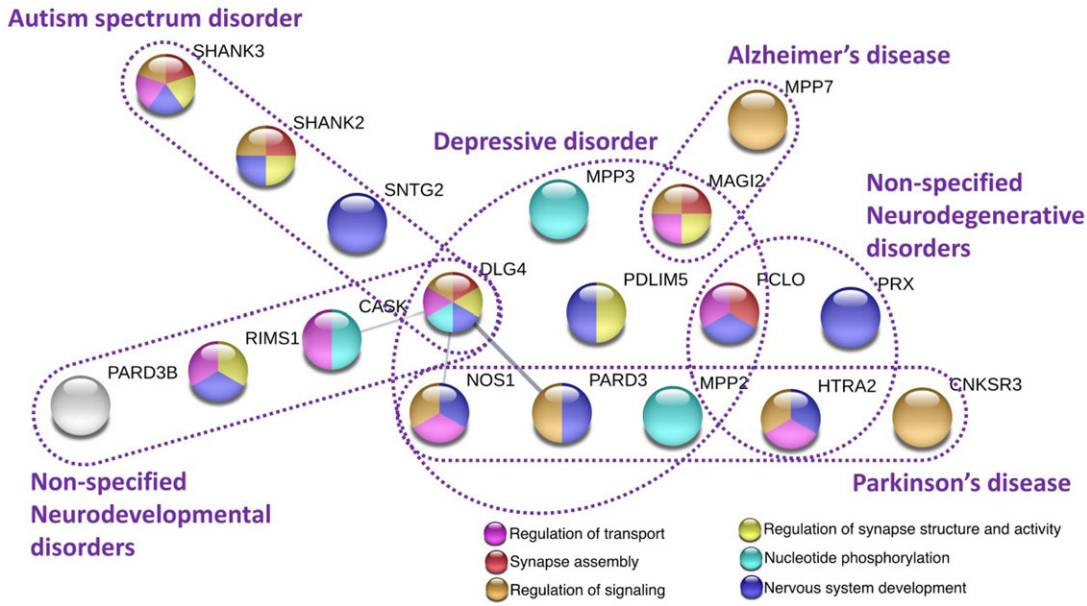


Figure 4. A selection of PDZ-containing proteins involved with neurological diseases. Proteins marked with circles are colored according to biological process GO term enrichment. Lines between proteins represent experimental evidence of the protein–protein interaction—the thicker the line, the higher the confidence of the evidence. Mapping was done using STRING database.

4.1.2. Targeting PDZ Domains of Postsynaptic Density Protein 95/Synapse-Associated Protein 90

PSD-95 is a master scaffold protein and is one of the first proteins where PDZ domains were identified. Together with Shank3, SAP-90/PSD-95-associated protein 1 (SAPAP1) and SynGAP, PSD-95 represents the core of synaptic scaffolding.[100] PSD-95 is highly expressed in the adult mouse brain, with highest expression levels in the hippocampus, cortex, and olfactory bulb.[101] In neurons, PSD-95 is highly enriched in synaptic regions, especially in the postsynaptic density (PSD).[102]

PSD-95 encoding gene (*DLG4*) is found on chromosome 17 (17p13.1), spanning approximately 30 kb, and is associated with neurological disorders as previously discussed. Our search in the databases mentioned above has shown that PSD-95 is associated with several neurological disorders, including schizophrenia, major depressive disorder (MDD), and autism spectrum disorder (ASD) (Figure 4 and Table S3, Supporting Information). Variations in *DLG4* have been associated with schizophrenia[103,104] and it was recently shown that epigenetic editing of *DLG4* could rescue memory deficits in a mouse model of Alzheimer's disease.[105] PSD-95 has also been found to have higher expression levels in the lateral amygdala in patients with MDD, compared to healthy individuals,[106] while a recent study showed no difference in epigenetic factors in the prefrontal cortex and hippocampus.[107] In addition, several variants in *DLG4* have been found in cancer patients; in total 21 different mutations have been found in the PDZ domains of PSD-95 (Table S3, Supporting Information, DLG4).

Three isoforms of PSD-95 have been characterized, which result from alternative splicing in the N-terminal region preceding the PDZ domains (Uniprot: Q62108 Mouse, P78352 Human). The canonical isoform of PSD-95 is a 724 aa protein, with a 64 aa N-terminal region, while isoform 2 (PSD-95β) contains 767 aa including a 107 aa N-terminal region, and isoform 3 (PSD95γ) is composed of 721 aa with a 61 aa N-terminus (Uniprot: P78352). While PSD-95 contains palmitoylation sites at C3 and C5, PSD-95β contains an N-terminal L27 domain (**Figure 5**a).[20,108,109]

Several post-translational modifications (PTMs) have been found to regulate the structure and function of PSD-95. Among these, palmitoylation as previously described, as well as several phosphorylations, have been found to influence the function, localization, and mobility of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs).[110–112] Together with synapse-associated protein 102 (SAP-102), postsynaptic density protein 93 (PSD-93), and SAP-97, PSD-95 is a core member of the MAGUK superfamily (Figure S3, Supporting Information), with a common domain topology of three PDZ domains (PDZ1-3), an SH3 domain, and a GK domain, as described in Section 2.

The primary role of PSD-95 is to stabilize and anchor membrane protein complexes in the synaptic membrane, and in the case of AMPARs and NMDARs, the direct or indirect association clusters these receptors into larger signaling complexes (Figure 5c). The interaction between PSD-95 and the NMDAR is primarily mediated by the direct interaction of the C-terminus of the GluN2B subunit and PDZ1 or PDZ2 of PSD-95 (Figure 5c,d). Furthermore, the PSD-95/NMDAR complex is stabilized by the interaction between PSD-95-PDZ3 and the NMDAR auxiliary subunit Neuropilin and tolloid-like protein 1 (NETO-1). Similarly, PSD-95 interacts with the AMPAR complexes through binding to the AMPAR auxiliary subunits, such as the voltage-dependent calcium channel γ-2 subunit (CACNG2), also known as transmembrane AMPA receptor regulatory protein γ2 (TARP γ2 or stargazin), which binds predominantly to PSD-95-PDZ3 (Figure 5c). Furthermore, PSD-95 holds a key role in the

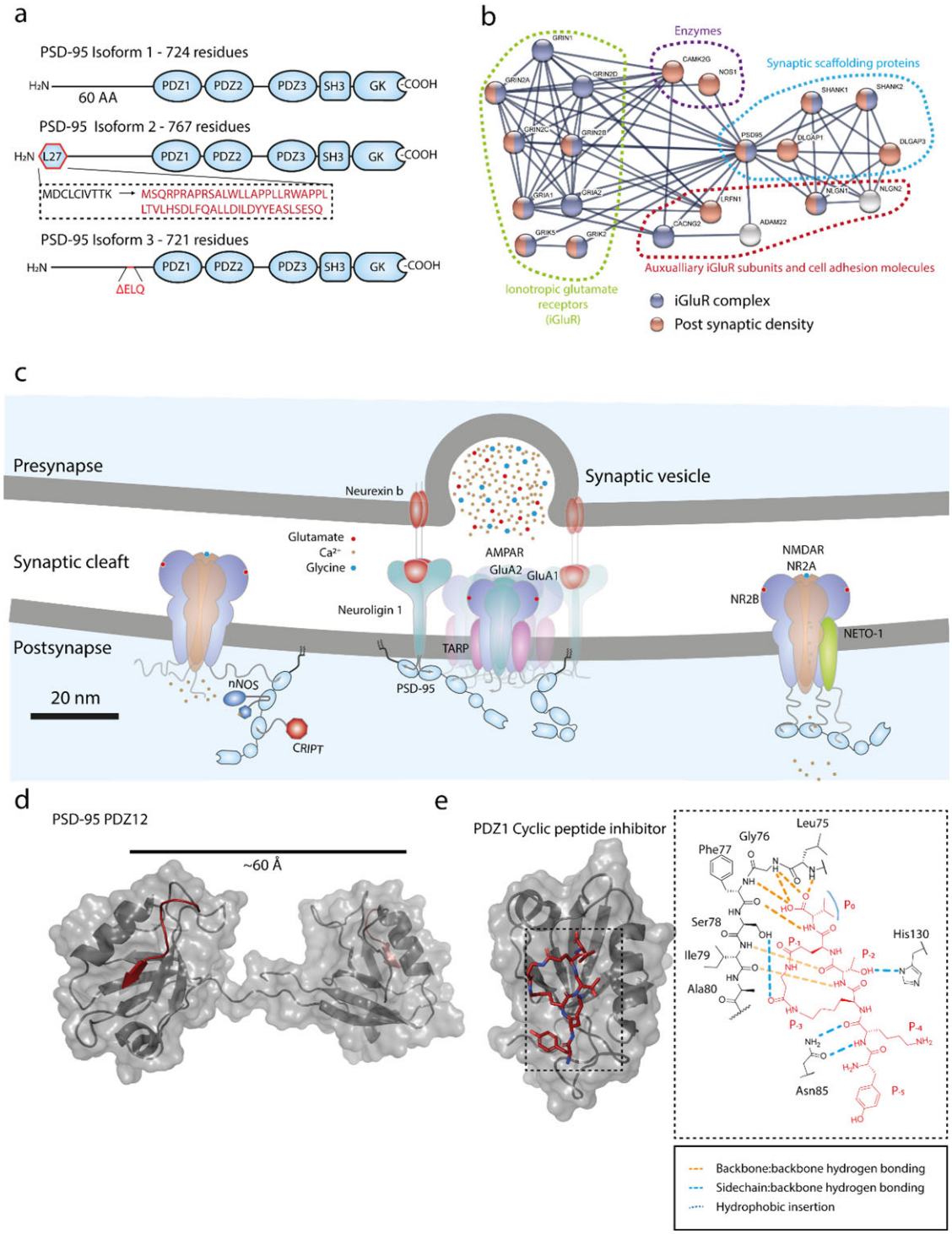


Figure 5. a) Domain organization of PSD-95 (Uniprot: P78352) and its splice variant derived isoforms. b) A protein interaction network (STRING) showing a selection of 20 proteins (highest confidence score) interacting with PSD-95 shows high interconnectivity between the different proteins in their respective groups. Made using STRING database information, analysis, and visualization tools. c) Graphical illustration of selected membrane protein/PSD-95 PDZ interactions in the postsynaptic density. d) Structure of PSD-95 PDZ1-2 in its double Cypin ($_{NH2}$-QVVPFSSSV-$_{COOH}$) occupied state shows parallel orientation of the PDZ1 and PDZ2 binding pocket (PDB: 2KA9). e) Structure (left) and hydrogen bonding network (right) of cyclic lactam-containing peptide ($_{NH2}$-YK-c[KTE(βA)]-V-$_{COOH}$) insertion into PDZ1 of PSD-95 displaying additional hydrogen bonds compared to canonical type II ligand insertion (PDB: 1RGR).

nNOS-mediated NO production following the abnormal synaptic Ca^{2+} influx resulting from ischemic stroke.[113] nNOS binds primarily to PDZ2 of PSD-95 through a noncanonical insertion of a β-hairpin motif in the binding pocket of PDZ2[114] (Figure 5c). The interaction between nNOS and PSD-95 ensures that nNOS and NMDAR are in close proximity, where Ca^{2+} influx under normal conditions leads to NO generation, which is required for further signaling. However, under excitotoxic conditions, massive calcium influx through the NMDAR activates nNOS, which leads to the generation of toxic levels of NO.[115–117]

There has therefore been a keen interest in disrupting the ternary PSD-95/nNOS/NMDAR complex, representing a potential novel mode of action for the treatment of ischemic stroke. Currently, many inhibitors have been designed targeting the PDZ domains of PSD-95,[118,119] with multiple promising lead candidates.[120,121] A number of small-molecule inhibitors (**Figure 6**, **Table 2**) targeting PSD-95-mediated interactions have been identified. Screening of a 150 000 compound library identified IC87201 (2-((1H-benzo[d][1,2,3]triazol-5-ylamino)methyl)-4,6-dichlorophenol) as an inhibitor of the PSD-95/nNOS interaction with an IC_{50} of 31 μM.[122] IC87201 was examined in vivo for nociceptive effects in mice after intrathecal (i.t.) administration and showed a significant pain reducing effect at 100 pmol in the presence of NMDA, with no effect in the absence of NMDA. Furthermore, IC87201 was tested in the chronic constriction injury (CCI) model of neuropathic pain, and displayed an alleviating effect on pain perception, and similar effects were seen when administering a variant of nNOS fused to the cell penetrating peptide (CPP) Tat.[122] A structure–activity relationship (SAR) study of IC87201 leads to the discovery of ZL006,[123] which was shown to be an inhibitor of the nNOS/PSD-95 interaction following glutamate/glycine treatment with an IC_{50} value of 82 nM in cultured cortical neurons. ZL006 was shown to ameliorate cerebral ischemic brain damage in mice when administered after middle cerebral artery occlusion (MCAO).[123] Later studies have shown promising effects of ZL006 in results from forced swimming tests[124] and regenerative repair following transient MACO (tMCAO)-induced stroke,[113] while recent studies have raised doubts about the rapid-onset antidepressant effects of ZL006 in mice.[125]

Due to a relatively poor cellular uptake of ZL006, the compound was reformulated and encapsulated in liposomes conjugated with polyethylene glycol (PEG) and T7, which targets transferrin receptors and enhance uptake.[126] The T7-P-ZL006 formulation increased the cellular uptake compared to ZL006 and T7-P-ZL006 showed a decrease in infarct volume following MCAO after i.v. injection. In further studies, it was shown that dual conjugation of T7 and stroke homing peptide (SHp) to the ZL006-containing liposomes increased the accumulation of the T7-SHp-ZL006 liposomes in the affected brain area following MCAO and reduced the infarct volume further compared to ZL006 and T7-P-ZL006.[127]

Although ZL006 shows promising neuroprotective effects, the specific molecular mechanism is still debated. It was originally suggested that ZL006 binds to the nNOS β-finger, thus preventing the nNOS β-finger from binding to PDZ2 of PSD-95.[123] However, a recent study showed that ZL006 does not bind the PDZ domains of PSD-95 or nNOS, and that ZL006 does not inhibit the PSD-95/nNOS complex.[128] Thus, ZL006 might not be a direct inhibitor of the PSD-95/nNOS complex, but acts through a yet unknown mechanism. In addition to IC87201 and ZL006, compounds such as tramiprosate (homotaurine, 3-amino-1-propanesulfonic acid), 4-PPBP (4-phenyl-1-(4-phenylbutyl)-piperidine), and honokiol have been proposed to inhibit the NMDAR/nNOS/PSD-95 ternary complex, but in all cases the specific mechanism remains unclear.

Due to the general low affinity of small molecules targeting PDZ domains, the most viable approach for designing inhibitors of PDZ domains seems to be the development of inhibitory peptides and peptidomimetics (Figure 6). Several studies have identified the key amino acids required for binding to PSD-95 and attempted to design and modify short peptide fragments for increased stability and affinity.[129,130] In one example, the hexamer CRIPT peptide, YKQTSV, was used as a template and P_{-1} (Ser) and P_{-3} (Gln) were substituted to Glu and Lys respectively, to facilitate an intramolecular lactam cyclization (Figure 5e). This cyclization strategy increased the affinity for PSD-95 PDZ1 tenfold, improved the half-life of the peptide, and disrupted the interaction between PSD-95 and GluK2 in HEK293 cells.[131] An analogue of the CRIPT peptide, YKQTKV, was used as a template for parallel synthesis of 272 different peptides, modified at the P_{-1} Lys with various organic acids, which led to a ≈20-fold increase in affinity.[132]

The first peptide inhibitor of PSD-95, NA-1 (or Tat-NR2B9c), was developed to target the interaction between PSD-95 and nNOS, minimizing the excitotoxicity resulting from ischemic stroke.[133] NA-1 is a fusion peptide of the nine amino acid C-terminal residues of the GluN2B NMDAR subunit, KLSSIESDV, and the nuclear transcription activation protein-derived cell penetration 11-mer peptide Tat (Figure 6). NA-1 was found to reduce the infarct size in the tMCAO rat model.[133] It was later found, using proteomic analysis, that NA-1 interacts with 44 different proteins, but only the PSD-95 and nNOS were found to be essential for the NMDAR-induced cytotoxicity.[134–136] In a clinical phase II study, NA-1 reduced the number of infarcts in patients at risk for embolic strokes while undergoing endovascular brain aneurysm repair, while not reducing the infarct size.[137] NA-1 is currently being investigated in a global phase III clinical trial (ESCAPE NA-1/NA-1-007) for reducing global disability in subjects with a major acute ischemic stroke, as well as a second trial in Canada (FRONTIER/NA-1-005) in patients with acute cerebral ischemia.[138] If NA-1 is proven to be effective in stroke treatment and is approved for treatment, it paves the way, not only for novel strategies in stroke treatment, but also for other treatment strategies targeting intracellular PPIs in general and PDZ domain proteins in particular.

Following the discovery of NA-1, several studies and approaches have investigated optimizing the endogenous C-terminal peptide, aiming to increase affinity as well as improve stability and selectivity. The structures of the first two PDZ domains of PSD-95 showed that the binding pockets of PDZ1 and PDZ2 are very similar.[70] This was exploited to develop a PDZ1-2 tandem-specific dimeric PSD-95 ligand, bridging two Kv1.4-derived decamer peptides (CSGSAWETDV) through a disulfide bond (Figure 6), which improved the affinity ≈20-fold relative to the monomeric ligand.[70] This approach was further developed, first showing that a pentameric GluN2B-derived peptide, IETAV, displayed WT affinity toward PSD-95,[129] and

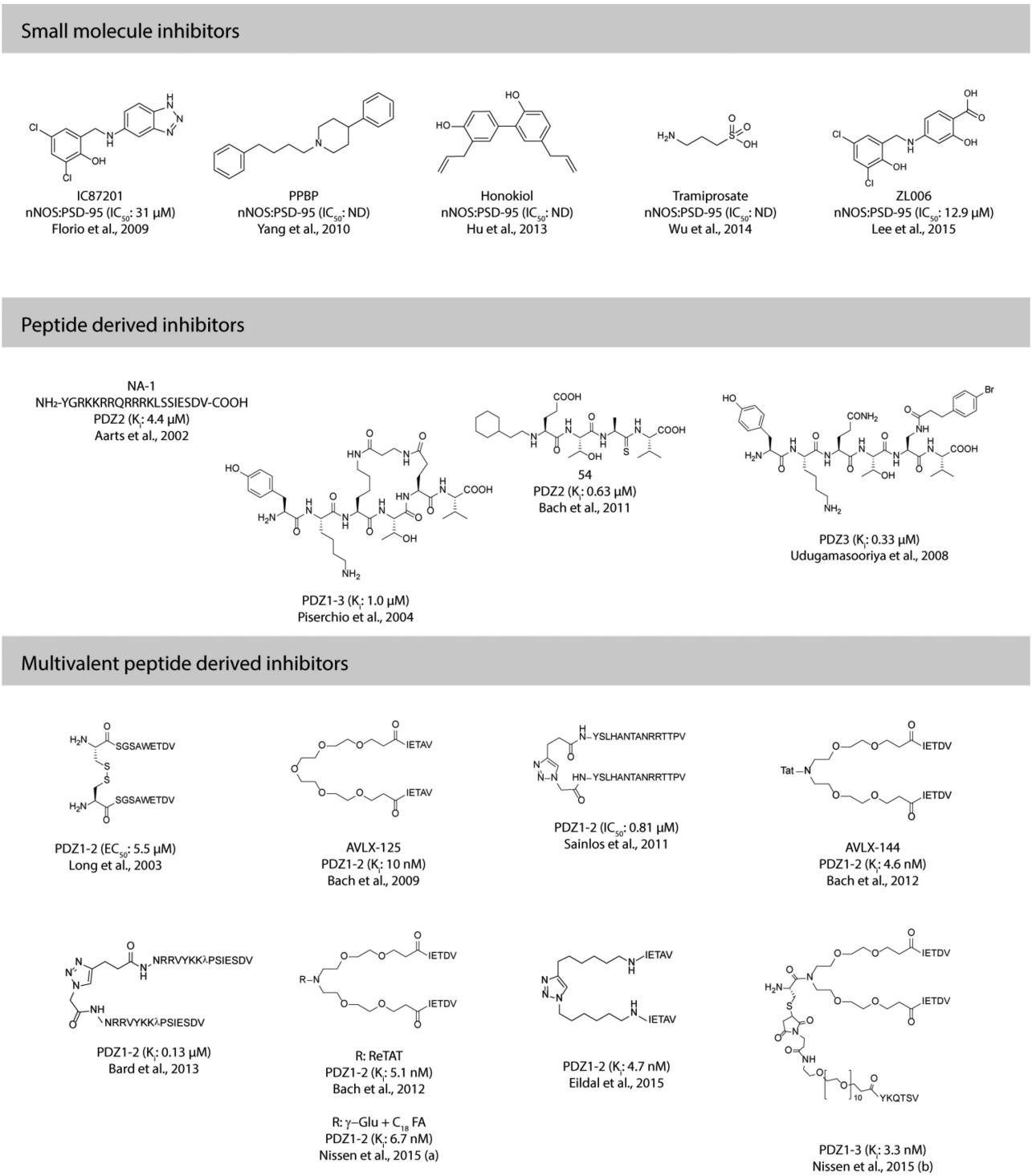


Figure 6. Structures and binding affinities for inhibitors targeting the PDZ domains of PSD-95. K_d and K_i values are given if otherwise not stated. ND, not determined.

subsequently showing that crosslinking the two peptides with a PEG linker, with optimized length (UCCB01-125, Figure 6) resulted in an affinity of 10 nM, a 145-fold increase relative to the monomeric peptide.[139] Notably, it was shown that crosslinking with the PEG linker substantially prolonged the plasma stability, while later studies showed that UCCB01-125 could not permeate the blood–brain barrier (BBB). Thus, a modified PEG linker was developed,[121] which included a central amine handle where a Tat peptide, YGRKKRRQRRR, was attached. This addition rendered UCCB01-144 (or AVLX-144) BBB permeable,

Table 2. Affinity and indications for selected peptide inhibitors of PDZ-mediated protein–protein interactions.

Target	Inhibitor[a)]	Affinity	Indication[b)]	Status	References
PSD-95/nNOS	"IC87201"	31 μM (IC_{50})	Pain, stroke	Preclinical	[122]
PSD-95/nNOS	"ZL006"	82 nM/13 μM (IC_{50})	Pain, stroke	Preclinical	[123,335]
PSD-95/nNOS	"Tramiprosate"	–	Stroke	Preclinical	[336]
PSD-95 PDZ12/nNOS	"UCCB01-144"	4.6 nM (K_i)	Stroke	Preclinical	[121]
PSD-95 PDZ12/nNOS	"NA-1"	4.6 μM (K_i)	Stroke	Phase III trial	[120]
PSD-95 PDZ3	YKQTSV analogs	0.3-2 μM(K_d)	NA	Development	[132]
PICK1	"FSC231"	10 μM (K_i)	Pain, Stroke	Development	[170]
Shank3	Tetrahydroquinoline analogs	10-70 μM (K_i)	NA	Development	[189]
Shank3	GSSGDPAWDETNL and multimeric analogs	8-41 μM (K_d)	NA	Development	[193]
Disheveled 1	"J01-017a"	1.5 μM (K_i)	Cancer	Preclinical	[215]
Disheveled 2	KWYGWF	0.7 μM (K_i)	Cancer	Preclinical	[42]
Scribble PDZ1	RSWFETWV	0.7 μM (K_d)	Cancer	Development	[239]
Syntenin PDZ12	$(CGSDKE\Phi\Phi V)_2$	0.2 μM (K_d)	Cancer	Preclinical	[337]
CAL	cyclo(CRRRRFWQC)TRV	0.5 μM (K_d)	Cystic fibrosis	Development	[300]

[a)] Capital letters indicate standard one-letter amino acid abbreviation for peptide inhibitors, except for Φ which indicates naphthyl-alanine. Compound names are shown quoted; [b)] Therapeutic indication. NA, not available.

and increased affinity further to 4.6 nM, a 1000-fold affinity increase compared to the corresponding monomeric peptide and NA-1.[121] UCCB01-144 was demonstrated to protect against ischemic brain damage in a mouse pMCAO model at a dose of 3 nmol g^{-1} given i.v. and improved neuromuscular function. Surprisingly, NA-1 did not show significant effect, which later was shown to be a dose dependent, and NA-1 showed effect in mice at 10 nmol g^{-1}.[140] Subsequent studies have explored UCCB01-125 and UCCB01-144 further: First, a series of trimeric ligands were developed targeting all three PDZ domains of PSD-95.[141] Second, to increase the plasma stability of the dimeric ligands, the Tat sequence was replaced with various fatty acids, which substantially increased the half-life in rats relative to UCCB01-144.[142]

In later studies, other related approaches were used to develop dimeric ligands targeting PSD-95. A dimeric, Tat-conjugated variant of the C-terminal 15-mer peptide of the NMDAR GluN2A subunit (Figure 6), surprisingly could decouple PSD-95 from GluN2A without disrupting the PSD-95/GluN2B interaction. Neurons incubated with 5 μM of the ligand showed that NMDARs were more mobile and less synaptically located.[143] In a similar approach, a series of dimeric 15-mer peptides derived from the TARP $\gamma 2$ were developed, which resulted in a 25-fold affinity increase relative to the monomeric peptide, while a ≈20-fold increase in IC_{50} value was obtained for a dimeric ligand incorporating a Tat sequence.[144]

Taken together, the dimerization of monomeric peptides to target PSD-95 seems to be an attractive method of increasing both target affinity and plasma stability. Furthermore, it appears that the multimerization strategy is suitable for targeting both the tandem PDZ domains (PDZ1-2)[70,121,139,141–143,145] and non-tandem PDZ domain (PDZ3) of PSD-95,[144] though the affinity benefit of targeting the PDZ1-2 tandem seems higher. With NA-1 in phase III clinical trials and the dimeric ligand UCCB01-144 in late-stage preclinical studies, it will be exciting to see potential new stroke treatments and explore PSD-95 as a drug target. In addition, these serve as a proof-of-concept for the targeting of intracellular PPIs such as the PDZ domain interactions.

4.1.3. Targeting the PDZ Domain of Protein Interacting with C Kinase 1

PICK1 is a unique protein in the human genome, as it is the only protein that contains both an N-terminal PDZ and a bin-amphiphysin-Rvs (BAR) domain (**Figure 7**a,c). PICK1 was originally cloned as a protein kinase Cα (PKCα) binding protein found in multiple tissues and organs, most abundantly in the brain, endocrine tissues, and skeletal muscle tissue (Human Proteome atlas).[146,147] It is highly evolutionarily conserved from invertebrates to humans, which suggests that PICK1 has important biological functions. Through the PDZ domain, PICK1 interacts with a number of neurotransmitter receptors, transporters, and enzymes (Figure 7f), where in particular the interactions with the AMPAR[148–157] and the dopamine transporter (DAT) have received particular attention.[158–160] The BAR domain of PICK1 is a domain that facilitates dimerization of PICK1. The BAR domain is further believed to promote tetramerization and oligomerization of PICK1 (Figure 7a,b).[161,162] The PDZ domain of PICK1 interacts with both lipids and proteins. In PICK1, a Lys is positioned at αB1 (K83), which explains its promiscuity in binding to different C-terminal ligand classes, though it does favor class II ligands (Figure 7c,d).[38] The βB-βC loop in the PDZ domain of PICK1 contains a Cys-Pro-Cys motif proposed to promote PDZ-PDZ dimerization,[56] zinc binding,[163] and lipid binding.[54]

The BAR domain, on the other hand, is characterized by sensing curvatures of membranes and can influence synaptic localization and function of target proteins.[161,162,164–167] PICK1 has been demonstrated to possess multiple functions in the CNS, particularly in the regulation of the function of AMPARs, specifically the GluA2 receptor subunit, where PICK1

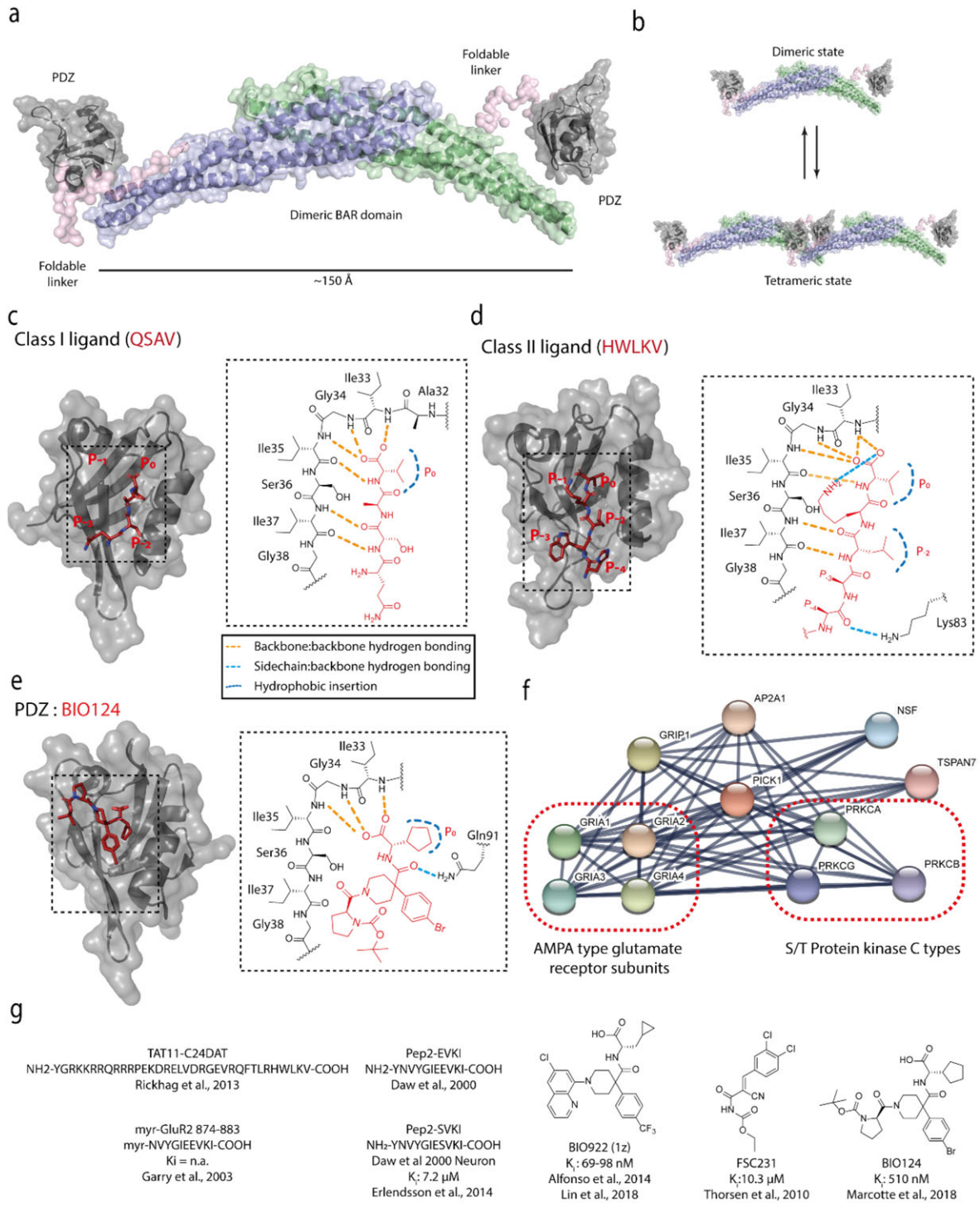


Figure 7. a,b) SAXS-derived model of dimeric and tetrameric PICK1 (SASDB: SASDAB8). c) Structure (PDB: 6BJN) of class I ligand ($_{NH2}$-QSAV-$_{COOH}$) binding to the PDZ domain of PICK1 shows canonical hydrogen bonding network, hydrophobic insertion of P_0, and side chain coordination of P_{-2} to αB1 Lys_{83}. d) Structure (PDB: 2LUI) of class II ligand (—HWLKV-$_{COOH}$) binding to the PDZ domain of PICK1 shows canonical hydrogen bonding network for a class II ligand, hydrophobic insertion of P_0 and P_{-2}; furthermore, the ligand displays partial coordination of P_{-1} Lys and C-terminal carboxylic acid and backbone/side chain interaction between P_{-4} carbonyl and Lys_{83}. e) Example of a class I mimicking small-molecule compound (BIO124, PDB: 6BJO), with coordination of the C-terminal carboxylic acid and P_0 amide hydrogen to the PDZ backbone Ile_{33}-Gly_{34}-Ile_{35} loop, and hydrophobic insertion of the P_0 cyclopropane ring; the interaction is further stabilized by hydrogen bonding between P_{-1} carbonyl and Gln_{91} side chain. f) A protein interaction network showing a selection of proteins (highest confidence score) interacting with PICK1 shows high interconnectivity between the different proteins in their respective groups. Made using STRING database information, analysis, and visualization tools. g) Structures and binding affinities for inhibitors targeting the PDZ domain of PICK1 and their respective affinities, if available.

is involved in the endocytosis of AMPARs during long-term depression (LTD) and regulation of AMPAR trafficking during the expression of synaptic plasticity.

PICK1 has been suggested to play a role in a range of neurological disorders, in particular, chronic pain, but also epilepsy, stroke, Parkinson's disease, and schizophrenia (Table S3, Supporting Information).[168] This has also led researchers to pursue inhibitors of PICK1 as a potential therapeutic modality and, as with other PDZ-containing proteins, the C-terminal peptide of the interacting proteins can be used as starting points (Figure 7c,d).

The first small-molecule inhibitor of the PDZ domain in PICK1, FSC231 ((E)-ethyl 2-cyano-3-(3,4-dichlorophenyl)acryloylcarbamate), was identified by a screening of ≈44 000 small molecules (Figure 7g).[169,170] This screening identified a couple of different small-molecule inhibitors with low μM affinity, where the most potent analogue, seemed to be an irreversible inhibitor.[169] Thus, FSC231 was pursued further and shown to bind PICK1 with a K_i of 10.1 μM, that is, in the same range as endogenous peptides, and FSC231 did not inhibit other PDZ-containing proteins such as PSD-95 and GRIP1. Moreover, it was shown that FSC231 both inhibited co-immunoprecipitation of the GluA2 subunit of AMPARs in cultured hippocampal neurons, and influenced recycling of GluA2 after internalization. Notably, it was also shown that FSC231 blocked the expression of both long-term depression and long-term potentiation in hippocampal cornu ammonis 1 (CA1) neurons from acute slices, which is in accordance with the inhibition of the bidirectional function of PICK1 in synaptic plasticity. In subsequent studies, an SAR study of FCS231 was performed, where a number of analogues were synthesized; however, only leading to a relatively modest improvement in affinity.[171]

Researchers at Biogen Idec have similarly pursued small-molecule inhibitors of PICK1 and have published different small molecules, including BIO922 and BIO124, which share a common chemical scaffold and all include a carboxylic acid and a closely located amide bond that coordinates binding to the carboxylate binding loop of the PICK1 PDZ domain (Figure 7g).[148,149] BIO922 was described as identified through structure-based design and has a K_i of 98 nM in blocking full length PICK1 and also a selectivity toward PICK1 versus PSD-95 and GRIP. The compound was employed in a mechanistic study examining how amyloid beta (Aβ) produces synaptic depression by enhancing endocytosis of AMPARs and it was demonstrated that BIO922 blocks the effects of Aβ synaptic transmission.[172] Another related compound, BIO124, which displayed an IC_{50} = 0.51 μM, was used in a structural study and a co-crystal X-ray structure demonstrated that BIO124 binds in the carboxylate binding loop of the PICK PDZ domain (Figure 7e,g).[149] In a recent study, the discovery of BIO922, BIO124, and related molecules were described in more detail.[148] High-throughput screening of 273 000 compounds was performed, which identified a scaffold, which was subsequently used in an SAR study, which identified BIO922 as the most potent inhibitor.[148]

The first peptide inhibitors of the PICK1 PDZ domain were Pep2-EVKI and Pep2-SVKI, which targeted the interaction between the AMPAR subunits GluA2/3 and PICK1 (Figure 7g).[173] Both peptides could efficiently displace the interaction between PICK1 and GluA2 in COS7 cells, but while Pep2-SVKI was found to inhibit LTD, Pep2-EVKI did not show an effect on LTD. Furthermore, Pep2-SVKI required PKC function to block LTD in hippocampal CA1 neurons.[173] Later studies have shown that Pep2-EVKI could reduce the reinstatement of cocaine seeking through decoupling of the AMPAR C-terminal/PICK1-PDZ interaction after intra-accumbal shell administration.[174]

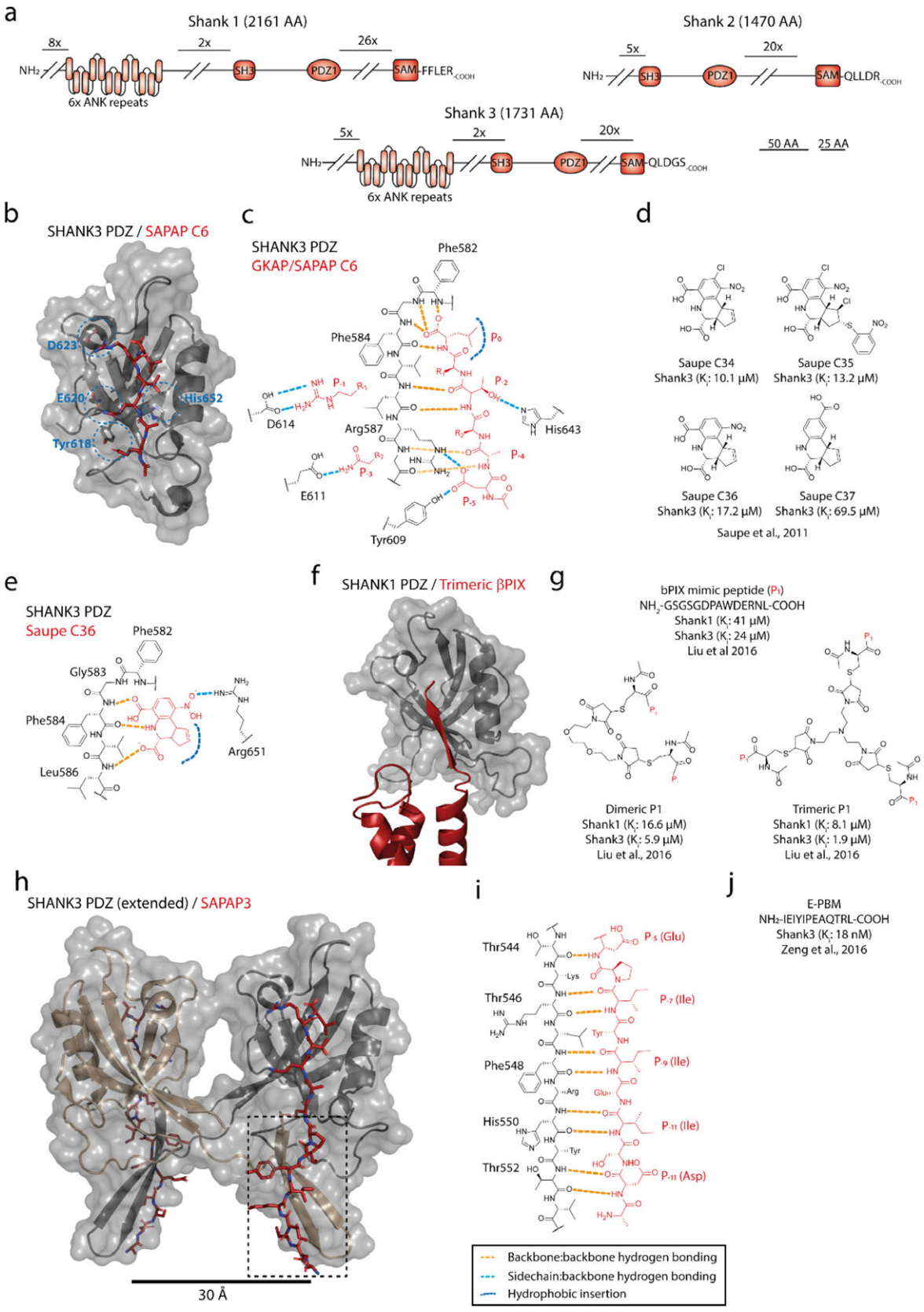
Further development of Pep2-EVKI and Pep2-SVKI was carried out by N-terminal myristylation, producing myr-Pep2-EVKI and myr-Pep2-SVKI, in order to increase the cell permeability and the plasma half-life of the peptides.[175] The involvement of PICK1 in chronic pain was also underlined after i.t. administration of myr-Pep2-EVKI, which showed a significant effect in thermal hyperalgesia and mechanical allodynia following CCI.[175] PICK1 has also been implicated in trafficking of DAT and is thereby implicated in maintaining the dopamine homeostasis.[158,176]

To elucidate the importance of the interaction between PICK1 and DAT, the 24 C-terminal residues of DAT, which contains both a PICK1 binding site at the extreme C-terminal and a Ca^{2+}/calmodulin-dependent protein kinase IIα (CamKIIα) binding site upstream of the PICK1 binding site, was fused to the Tat CPP motif to render the peptide cell permeable (Tat-DATC24). This peptide blocked the interaction between PICK1 and DAT, as well as the interaction between CamKIIα and DAT, altogether reducing the amphetamine-induced dopamine efflux in striatum after local administration. Furthermore, the peptide significantly reduced the amphetamine-induced hyper locomotor activity after i.v. injection, suggesting that the administration of a PICK1 PDZ inhibitor will reduce the DAT-mediated effect of amphetamine.[176]

PICK1 is a multifaceted protein that is involved in several essential pathways for synaptic plasticity and neurotransmitter homeostasis. Inhibitors of PICK1 have, with success, been investigated in relation to several indications. Nevertheless, the promiscuous role of PICK1 could suggest that targeting of PICK1 with a systemic intervention strategy could have unforeseen consequences in the PICK1-dependent homeostatic interplay between ion channels, neurotransmitter transporters, and kinases.

4.1.4. Targeting the PDZ Domain of SRC Homology 3 and Multiple Ankyrin Repeat Domains 3

The family of proteins encoded by the *SHANK* genes, and mutations therein, have been implicated in several neurological disorders including ASD, schizophrenia, and intellectual disability (Table S3, Supporting Information). The *SHANK* genes encode Shank1, Shank2, and Shank3 (**Figure 8**a), where Shank3 is the best-characterized protein. Variations in the *SHANK3* gene have been found in several patients suffering from diverse neurological disorders. A direct association between Shank3 and disease have been found in patients with the rare genetic disorder, 22q13.3, which leads to Phelan-McDermid syndrome.[177] Partial or complete deletion in chromosome 22q13.3, encoding Shank3 (haploinsufficiency), causes late motor development, highly delayed speech, and intellectual disability.[178,179] While decreased Shank3 expression leads to lowered signal transduction, the overexpression of Shank3 was found to lead to manic behavior and epilepsy in mice, and hyperkinetic disorders in



mice and potentially in human patients.[180] Shank3 is involved in many signaling pathways in the CNS, including transport regulation, regulation of signaling, synapse assembly, synapse structure and function (Table S2, Supporting Information).

The Shank proteins are described as key scaffolding proteins due to their role as linkers between the receptor scaffolding proteins and the cytoskeleton scaffolding proteins. The Shank proteins are translated in a variety of isoforms resulting from both intragenic promoters and alternative splicing exons (Uniprot: Q9BYB0). Furthermore, the *SHANK* genes seem to be further regulated by epigenetic factors.[181] The Shank proteins are all expressed in the excitatory synaptic regions, where they are crucial for proper synaptic transmission and function.[182] The canonical isoform of Shank3 (Uniprot: Mouse: Q4ACU6-1, Human: Q9BYB0-1), contains six ankyrin repeats, an SRC homology 3 (SH3) domain, a PDZ domain, a proline rich region (PRO), and a sterile alpha motif (SAM) (Figure 8a). Although 30 different mutants have been found in the Shank3 gene, and at least three have been shown to associate to psychiatric diseases, none are positioned within the PDZ domain of Shank3. The variant Arg536 (R536W) is positioned in the linker region between the SH3 domain and the PDZ domain of Shank3, but does not interfere with the binding of C-terminal ligands nor changes the overall structure of the SH3-PDZ tandem.[183] Furthermore, several PTM sites have been predicted; however, none in the PDZ domain. The PDZ domains of the Shank proteins are members of the class I PDZ domains, with a His positioned in the αB1 helix, which is proposed to bind primarily C-terminal ligands with a S/T-x-φ motif (Figure 8b,c). The PDZ domain of Shank3 interacts directly with the GluA1 subunit of AMPARs[184] and SAPAP1.[185–188] SAPAP1 links Shank3 to the NMDARs through an interaction with the GK domain of PSD-95, which binds to NMDAR through its PDZ domains, as previously described. It was recently shown that the interaction between SAPAP3 and the PDZ domain of Shank3 was highly dependent on an N-terminal extension of the PDZ domain, which enhanced the peptide affinity significantly to 18 nM for SAPAP3 C15 (Figure 8h–j).[40]

The implication of Shank3 in neurological disorder has promoted the development of inhibitors toward its PDZ domain. A set of compounds was discovered by screening of a small-molecule library[189,190] containing a tetrahydroquinoline carboxylate core structure (Figure 8d,e). The compounds were shown to displace the SAPAP1 hexapeptide with modest affinity in the 10–70 μM range. Interestingly, it was showed that inhibitor affinity was highly dependent on the oligomeric state of Shank3,[191] and that βPIX (p21-activated kinase interacting exchange factor) forms trimer complexes that can increase the local proximity and thereby the local concentration of the Shank3 binding region, thus increasing the affinity (Figure 8f).[192] This inspired the design and synthesis of a series of monomeric, dimeric, and trimeric peptides targeting the PDZ domain of Shank1; however, peptide multimerization exhibited only a modest affinity increase (Figure 8g).[193]

4.2. PDZ Domains as Drug Targets in Cancer

4.2.1. Involvement of PDZ Domain Proteins in Cancer

PDZ-containing proteins are known to play an important role in cancer, from tumor formation to metastasis, especially through canonical interactions of their PDZ domains in signaling pathways.[194] In fact, 145 of 151 PDZ domain proteins have been suggested to be associated with cancers of various forms, especially skin, uterine, stomach, and lung cancers (Table S3, Supporting Information).[195–198] Many cancer-causing mutations have been detected in PDZ domains, with the majority of amino acid substitutions being a change of Arg or Gly to a different amino acid. The majority of mutations appear in PDZ domains of GRIP1, membrane-associated guanylate kinase, WW and PDZ-containing proteins 1 and 2 (MAGI1 and MAGI2), InaD-like protein (PATJ), PDZ domain-containing protein 2 (PDZD2), and multiple PDZ domain protein (MPDZ/MUPP1). The mutations in MPDZ are twice as common as in GRIP1. Most of these proteins are present in cell–cell junctions (Figure 3), which are recognized to play a role in cancer.[199]

4.2.2. Targeting the PDZ Domains of Disheveled Proteins

Disheveled (Dvl, also known as disheveled homolog [Dsh]) was first discovered in Drosophila melanogaster in 1959, where a mutation in the disheveled gene (*DVL1*) caused improper wing and body hair location.[200] Later, Dvl2 and Dvl3 were identified and these three disheveled proteins make up the Dvl protein family.[201] Sequence alignment of the protein family reveals three conserved domain arrangements, including an N-terminal dishevelled/axin (DIX) domain, a central PDZ domain, followed by a C-terminal DVL/Egl-10/pleckstrin (DEP) domain, which recognize and bind short peptide motifs.[202] The Dvl proteins have later been identified as key mediators of the Wnt signaling pathways and thus important for embryonic and adult development.[203]

Figure 8. a) Domain organization of Shank family proteins (Uniprot: Q9Y566, Q9UPX8, Q9BYB0). b,c) Canonical insertion of a type I ligand ($_{ac}$-EANTRL-$_{COOH}$, red) into the PDZ domain of Shank3, with canonical hydrogen binding network, hydrophobic insertion of P_0 (Leu) and P_{-2} (Thr) coordination with His_{643}; furthermore, the interactions are supported by side chain interactions between P_{-1}(Arg)/Asp_{614} and P_{-3}(Gln)/Glu_{611}. d). Small-molecule inhibitors targeting the PDZ domain of Shank3. e) Hydrogen binding network of C36 (red) binding to the PDZ domain of Shank3, mimicking the backbone hydrogen bonding network of the P_0 and P_{-1} residues from canonical ligands, and hydrophobic insertion of cyclopentene moiety; the binding is further stabilized by the interaction between the nitro group (—NO_2) and Arg_{651}. f) Binding of trimeric β-PIX (red) to the PDZ domain of Shank3 shows that steric hindrance reduces the expected avidity effect expected from close proximity.[334] g) Monomeric, dimeric, and trimeric peptide analogues mimicking βPIX binding to the PDZ domain of Shank1 and Shank3. h) Structure of SAPAP C15 ($_{NH2}$-ADSIEIYIPEAQTRL-$_{COOH}$, red) binding to an extended variant of the PDZ domain of Shank3 shows ligand-induced PDZ-PDZ' dimerization (PDB: 5IZU). i) Hydrogen binding network of β-strand/β-strand coordination between SAPAP3 P_{-5} and P_{-14} (red) and the Shank3 PDZ domain extension accounts immense affinity enhancement and ligand specificity. j) Primary sequence of the SAPAP3 C15.

The *DVL1* gene is located on chromosome 1 [1p36.33] and encodes for a 75 kDa (695 aa) protein, while *DVL2* and *DVL3* are located on chromosome 17 [17p13.1] and 3 [3q27.1] and encode for a 79 kDa (736 aa) and 78 kDa (716 aa), respectively. Cross-species analysis shows that DVL1-3 are highly conserved and studies of mice with single *DVL* gene homologue knockout have shown phenotypes that range from defective social behavior to developmental defects, including abnormal heart, skeletal and cochlear morphologies, suggesting a unique role for each of the three single *DVL* genes. However, the analysis of double or triple *DVL* knockouts reveals novel and more severe phenotypes, suggesting an overlap or redundant functions of the *DVLs*.[204]

Dvl1-3 are key mediators of the Wnt signaling pathway, which is evolutionary conserved and initiates signaling cascades that are crucial for embryonic development and maintenance of adult tissue homeostasis by regulating cell proliferation, differentiation, migration, genetic stability, apoptosis and maintaining adult stem cells in a pluripotent state. The Wnt signaling is commonly divided into the β-catenin-dependent (canonical) and independent (noncanonical) pathway. In the canonical signaling pathway, Wnt ligands, which are secreted glycoproteins, bind to the N-terminal extracellular cysteine-rich domain of the G protein-coupled receptor (GPCR) family Frizzled (Fz).[205] The signal is propagated through Dvl by a direct interaction between the internal recognition motif (KTXXXW) located in the C-terminal region of Fz and the Dvl PDZ domain,[202] followed by polymerization of Dvl, mediated by the DIX domain. The Dvl polymers function as structural scaffold platforms to further facilitate the dissemination of the Wnt signal, which leads to the activation of β-catenin and T-cell factor (TCF)-dependent transcription of developmental genes and genes associated with tumorigenesis.[206–208] The noncanonical Wnt signaling pathway refers to a group of signaling pathways, which do not lead to β-catenin-dependent transcription. The two best-studied noncanonical signaling pathways, the planar cell polarity (PCP) and the Wnt-calcium pathways, are both initiated by Wnt binding to Fz followed by intracellular interaction with Dvl. However, the downstream interaction partners of Dvl and effectors differ between the two signaling pathways. Ultimately, the PCP pathway leads to a rearrangement of the cytoskeleton and thus polarization of the cell and the Wnt-calcium pathway regulates the intracellular release of calcium from the endoplasmic reticulum (ER).[209] Given the pleiotropic nature of the Wnt signaling cascades, it is not surprising that regulation of this pathway is associated with a variety of disease states, including fibrosis, neurodegeneration, and cancer (Table S3, Supporting Information).[203] Specifically, aberrant Wnt signaling has been reported in tumors from patients with hepatocellular carcinoma, hepatoblastoma, colorectal cancer, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma, and gastric cancer.[210] The impact of Wnt signaling on cancerogenesis of colorectal cancer is particularly well described and tightly connected to the loss of the protein adenomatous polyposis coli (APC),[211,212] a downstream effector of Dvl and regulator of tumor suppressor genes. Functional loss of APC results in constitutive activation of Wnt signaling and thus promoting transcription of genes encoding oncoproteins.[213]

Over the last decades, inhibition of the Wnt signaling pathway has emerged as an attractive intervention point for the treatment of cancer and several studies have identified inhibitors targeting various points along the pathway.[203] In particular, several small molecules and peptide-based inhibitors targeting Dvl have been described (**Figure 9**g). The first Dvl inhibitor targeting the PDZ domain, NSC668036, was identified through receptor-based virtual screening of the National Cancer Institute (NCI) small-molecule library[214] (Figure 9g). Later, the same group developed a more potent small-molecule inhibitor, J01-017a, ($K_d = 1.5$ μM) by combining 3D quantitative structure–activity relationship (3D-QSAR) analysis and structural studies.[215] Another Dvl-PDZ inhibitor, FJ9, was designed based on an indole-2-carbinol scaffold, and shown to block the interaction between the C-terminal of Fz and Dvl ($K_d = 29$ μM) and to suppress tumor growth in vivo.[216] Next, a series of indole-2-amide-based Dvl inhibitors was described, with the aim to mimic the side chain interaction of fourth amino acids of native PDZ ligands. The optimized inhibitor, 6e, showed an IC_{50} value of 23 μM.[217] The small-molecule inhibitor, 3289-8625, was developed by a combination of in silico screening and structural analysis,[218] binds to Dvl with a K_d of 11 μM, has cell permeable properties, and inhibits Wnt signaling pathways.[217] Nonsteroidal anti-inflammatory drugs (NSAIDs) are best known for its pain-relieving properties by inhibiting cyclooxygenase (COX) enzymes, but evidence suggests that NSAIDs also affect the Wnt signaling pathway. One such example is sulindac (Clinoril, Figure 9e,f), which suppresses the canonical Wnt pathway in breast cancer, lung cancer, and colon cancer cell lines by binding to the PDZ domain of Dvl with an affinity of $K_d = 11$ μM.[219] In another study, efforts in identifying small-molecule inhibitors targeting the Dvl PDZ domain were based on screening commercially available compound libraries, which identified 20 hits, with several compounds containing an indole ring.[220] KY-02052 and KY-02061 were identified as the lead compounds. Although KY-02052 significantly increased nuclear β-catenin, the compound showed severe cytotoxicity in cell viability assays and was not pursued further. Thus, KY-02061 ($IC_{50} = 24$ μM) was shown to be the most active compound with less cytotoxic effects.[220] However, KY-02327 was later shown to have poor metabolic stability. To further develop KY-02327, 55 KY-02061 derivatives were constructed and KY-02327, containing a 2-aminoethyl piperidyl moiety at the carboxylic group, showed improved affinity ($IC_{50} = 3.1$ μM), stability, is orally active, and dose-dependently regulated the Wnt/β-catenin pathway. NMR experiments revealed that the binding site for KY-02327 is located in the canonical PDZ binding pocket, including three residues in the α2-helix (V318, R322, and V325).[220] A more recent effort in developing small molecules targeting the PDZ domain of Dvl[221,222] was initiated by generating a pharmacophore model, based on the X-ray crystal structure of Dvl PDZ/Dapper peptide complex and the NMR structure of Dvl PDZ/sulindac (Figure 9e,f).[219,223] The model was used to screen approximately 7 million virtual compounds and 19 initial hits were validated by surface plasmon resonance (SPR), which showed eight compounds that bind to Dvl PDZ. The four best hits all contained a phenoxyacetic acid group and with BMD4602 showing the highest affinity ($K_d = 11$ μM).[221] This study was followed by the generation of two new pharmacophore models based on molecular dynamic (MD) simulations of the Idax peptide (RKTGHQICKFRKC)–Dvl PDZ interaction and three known X-ray crystal structures of synthetic peptides binding to Dvl PDZ. A virtual library comprising 8 million compounds was

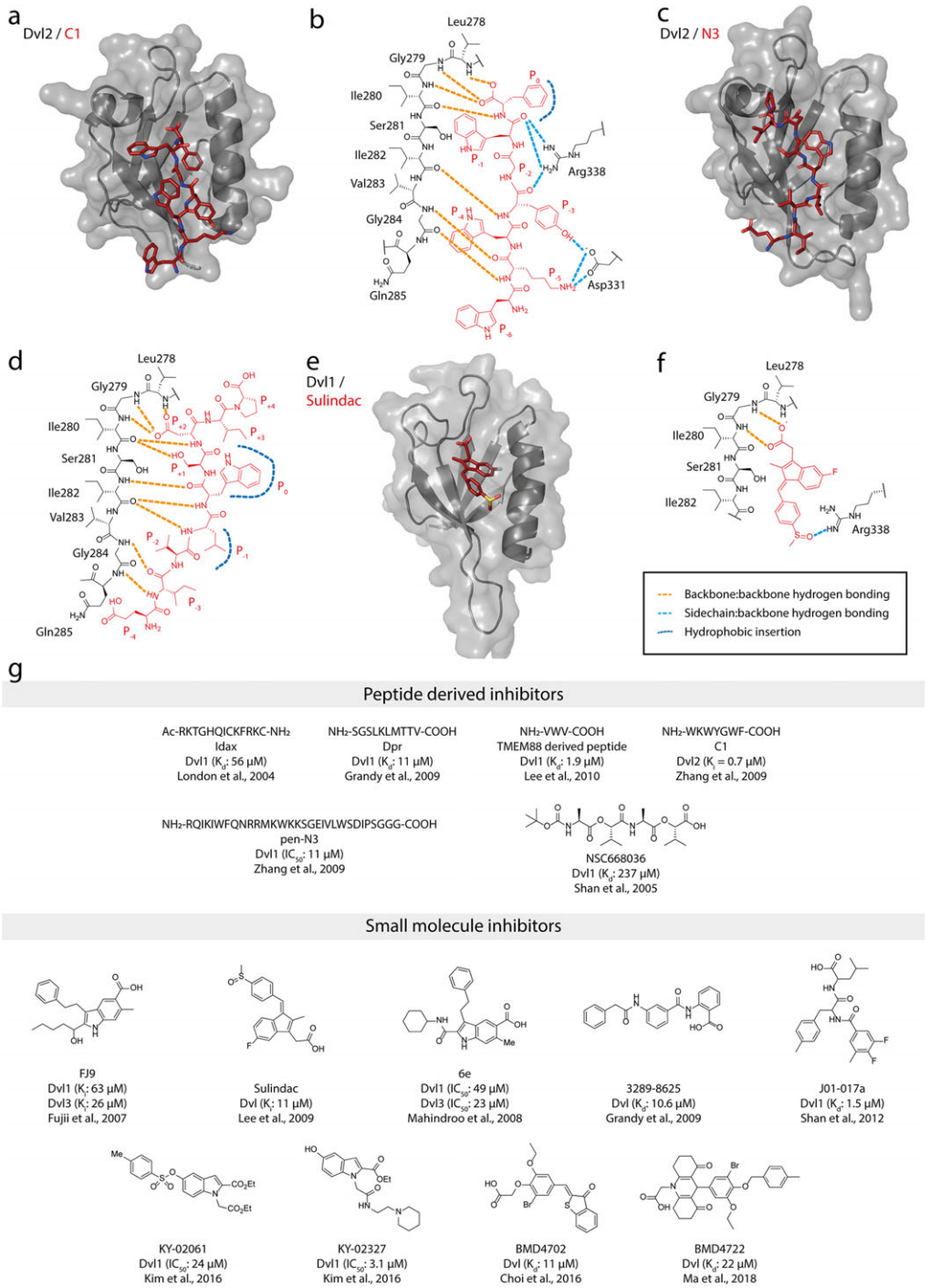


Figure 9. a) Structure of pseudo class II inhibitory peptide C1 ($_{NH2}$-WKWYGWF-$_{COOH}$, red) binding to the PDZ domain of segment polarity protein dishevelled homolog DVL-2 (Dvl2) (PDB: 3CBX). b) Hydrogen bonding network of C1 binding to the PDZ domain of Dvl2 show a higher extent of hydrogen bonding to the βB backbone than canonical binders, ending at P_{-6} amide hydrogen; furthermore, the interaction is stabilized by hydrogen bonding between P_{-1}/P_{-3} carbonyls and Arg_{338}, and side chain interactions between P_{-3} (Tyr)/P_{-5} (Lys) and Asp_{331}. c) Structure of noncanonical inhibitory peptide N2 ($_{NH2}$-GGEIVLWSDIP-$_{COOH}$, red) binding to the PDZ domain DVL-2 (PDB: 3CC0). d) Hydrogen bonding network of N3 binding to the PDZ domain of Dvl2 shows an upward shifted insertion of the ligand, with hydrophobic insertion of P_0 (Trp) and P_{-2} (Val). The backbone Leu_{278}-Gly_{279}-Ile_{280} loop is coordinating the P_{+2} (Asp) side chain, while P_{+1} (Ser) side chain is coordinated by the Ile280 carbonyl. The interaction is further strengthened by the backbone interaction between the amide hydrogen/carbonyl at Gly_{284} and the P_{-3} (Ile) carbonyl/amide hydrogen. e) Structure of a small-molecule sulindac (red) binding to the PDZ domain Dvl1 (PDB: 2KAW). f) Only few interactions are present in the sulindac binding to the Dvl1 PDZ domain; hydrogen bonds are present between the sulindac carboxylic acid and the Leu_{278}-Gly_{279}-Ile_{280} loop and the sulindac sulfoxide and Arg_{338}. The fluorinated indole ring system of sulindac inserts into the hydrophobic pocket between αB and βB, acting as the P_0 hydrophobic residues in canonical binders. g) Peptides, peptidomimetics, and small-molecule inhibitors targeting the PDZ domain of Dvl1-3.

examined and 16 virtual hits were identified and experimentally validated by fluorescence polarization (FP) assays. One compound, BMD4722, was confirmed as a PDZ domain binder (K_d = 22 μM). The binding mode of BMD4722 was validated by NMR and shown to involve residues Ile267, Ser268, Ile269 (located in the β2-sheet), Val323, and Arg325 (located in the α2-helix).[222]

Several peptide-based inhibitors for Dvl PDZ have been described, including three inhibitors which are based on the sequence of native Dvl PDZ interaction partners, and one inhibitor which is derived from phage display screening (Figure 9g). The native-derived peptide sequences are based on C-terminal regions of TMEM88, Dapper (Dpr), or Idax, and these peptide inhibitors have primarily been developed and used as molecular probes for the Wnt signaling pathway. Generally, most PDZ interactions only require the last three to four amino acids in the C-terminal tail of the ligand. However, the Dvl PDZ has been shown to interact with internal motifs. This is the case for Idax, which interacts with Dvl PDZ via a KTXXXI motif, and is therefore a negative regulator of the Wnt signaling pathway.[224] The Idax peptide, Ac-RKTGHQICKFFRKC-NH_2, binds to Dvl1 with a K_d of 56 μM, which is in the range of native PDZ–ligand interactions. The binding mode was verified by NMR studies, which also confirmed the peptide to be located in the groove between α2-helix and β2-sheet.[225] In 2002, the C-terminal region of Dpr, a Dvl-dependent regulator of normal vertebrate development via the canonical Wnt-signaling pathway, was shown to interact with Dvl.[223] Specifically, the last four amino acids of the C-terminal tail of Dpr, SLKLMTTV, were suggested to be required for the interaction.[223] The Dpr/Dvl canonical interaction was confirmed by NMR.[223] The interaction between the Dvl PDZ/Dpr derived peptide was later validated and determined the affinity to K_d = 11 μM[218] (Figure 9g). The researchers responsible for the development of the small-molecule Dvl inhibitors, FJ9 and J01-017a,[215,216] also designed peptide-based inhibitors. They started out by using a tripeptide template comprising VVV, which has been reported to have a β-strand-like structure in water. The interaction between PDZ Dvl and VVV was validated by NMR. Next, the amino acid side chain in position P_{-1} was examined for the improvement of the binding, and 18 proteinogenic amino acids at the P_{-1} positions in the VXV scaffold (X: any amino acid except Pro) were virtually introduced. The tripeptide VWV was simulated to have the highest binding energy and further validated by NMR and fluorescent binding experiments. The K_d of VWV to PDZ Dvl1 was determined as 2 μM, compared to 71 μM for VVV.[219] Later, the transmembrane protein 88 (TMEM88), comprising a VWV motif in the C-terminal, was identified as Dvl binder and shown to attenuate the Wnt/β-catenin signaling pathway.[226]

Phage display has also been used to identify peptide-based inhibitors for Dvl.[42,227] First, C-terminal phage-display peptide libraries comprising $>10^{10}$ unique members were used to identify ligands for Dvl2. Ten unique peptides were found and shown to have a preference for ligands comprising a $\Omega\phi$GWF C-terminal motif (Ω: hydrophobic residues, ϕ: aromatic residues, Figure 9a,b).[227] A second attempt used a phage display library comprising internal peptide ligands lacking the free C-termini, and identified 127 peptides that were grouped into three sequence families (N1-N3). Each subfamily contained a conserved Asp, which most likely substitutes for the free carboxylate on the C-termini.[42] The affinity and the binding mode of the three peptides (N1: WKDYGWIDGK, N2: SGNEVWIDGP, N3: EIVLWSDIP) binding to the Dvl2 PDZ domain were characterized by X-ray crystallography (Figure 9c,d) and FP. A detailed study of the Dvl2 PDZ revealed a flexible binding cleft, which can accommodate both linear peptide motifs as well as internal ligands. The lead peptide N3, with a K_d of 4.6 μM, was further validated by conjugating a CPP tag (antennapedia) and a fluorophore to the peptide, to enable intracellular location and visualization in HEK293S cells. Peptide N3 was readily internalized and inhibited canonical Wnt/β-catenin signaling in HEK293S in a dose-dependent manner, and was shown to be at least ten times more potent compared to compound FJ9.[42]

4.2.3. Targeting the PDZ Domains of Scribble

Scribble (*SCRIB*) was first discovered in Drosophila melanogaster as a tumor suppressor gene, as deletion of it caused aberrant proliferation and outgrowth of tissue.[228] Human Scribble is a 1630 aa protein comprising an N-terminal leucine-rich repeat (LRR) region followed by four PDZ domains and a flanking C-terminal tail (**Figure 10**a). The protein is involved in the establishment of cell polarity, cell migration, and tight junctions.[228,229] Dysregulation of Scribble has been shown to promote cell proliferation and tumorigenesis. Mammalian Scribble is part of an intracellular protein complex comprising disk large (Dlg1-5) and lethal giant larvae (Lgl1/2). This protein complex is located in the basolateral compartments of epithelial cells[230–233] and is required for the establishment of apical-basal cell polarity and growth control.[234] Disruption of apical-basal polarity leads to uncontrolled cell proliferation and thus stimulates the epithelial-to-mesenchymal transition (EMT), which is a major factor in cancer development.[235] In addition, Scribble has been shown to be part of the Hippo pathway, an evolutionarily conserved kinase signaling pathway involved in organ size control and tissue homeostasis.[236] Scribble interacts with Fat1, a transmembrane receptor and an upstream regulator of the Hippo pathway, via its PDZ domains. However, this interaction mechanism is not conserved across species. The C-terminal amino acid sequence of Fat1 in human, mouse, and zebrafish constitutes a class I PDZ binding motif (HTEV). This binding motif has been reported as responsible for the interaction between Scribble and Fat1. Drosophila Fat-like protein has also been reported to interact with Scribble. This interaction, however, is not dependent on the C-terminal PDZ binding motif (HTEV), suggesting that Scribble recognizes an internal epitope in Drosophila.[237] Scribble is also interacting with the protein β-PAK-interacting exchange factor (PIX), a guanine nucleotide exchange factor for the small GTPases Rac1 and Cdc42a (Figure 10c–e).[238] The interaction between the C-terminus of β-PIX (PAWDETNL) and the four PDZ domains of Scribble has been studied in detail. The X-ray crystal structures of the β-PIX peptide binding to PDZ1 and PDZ3 highlight how the binding preference and specificity is achieved (Figure 10d). The PDZ1 interaction is dependent on the side chains of Leu_0, Thr_{-2}, Glu_{-3}, and Trp_{-5}, while the primary determinant for the selectivity is Trp_{-5}. In PDZ1, the Trp_{-5} forms a hydrophobic interaction by

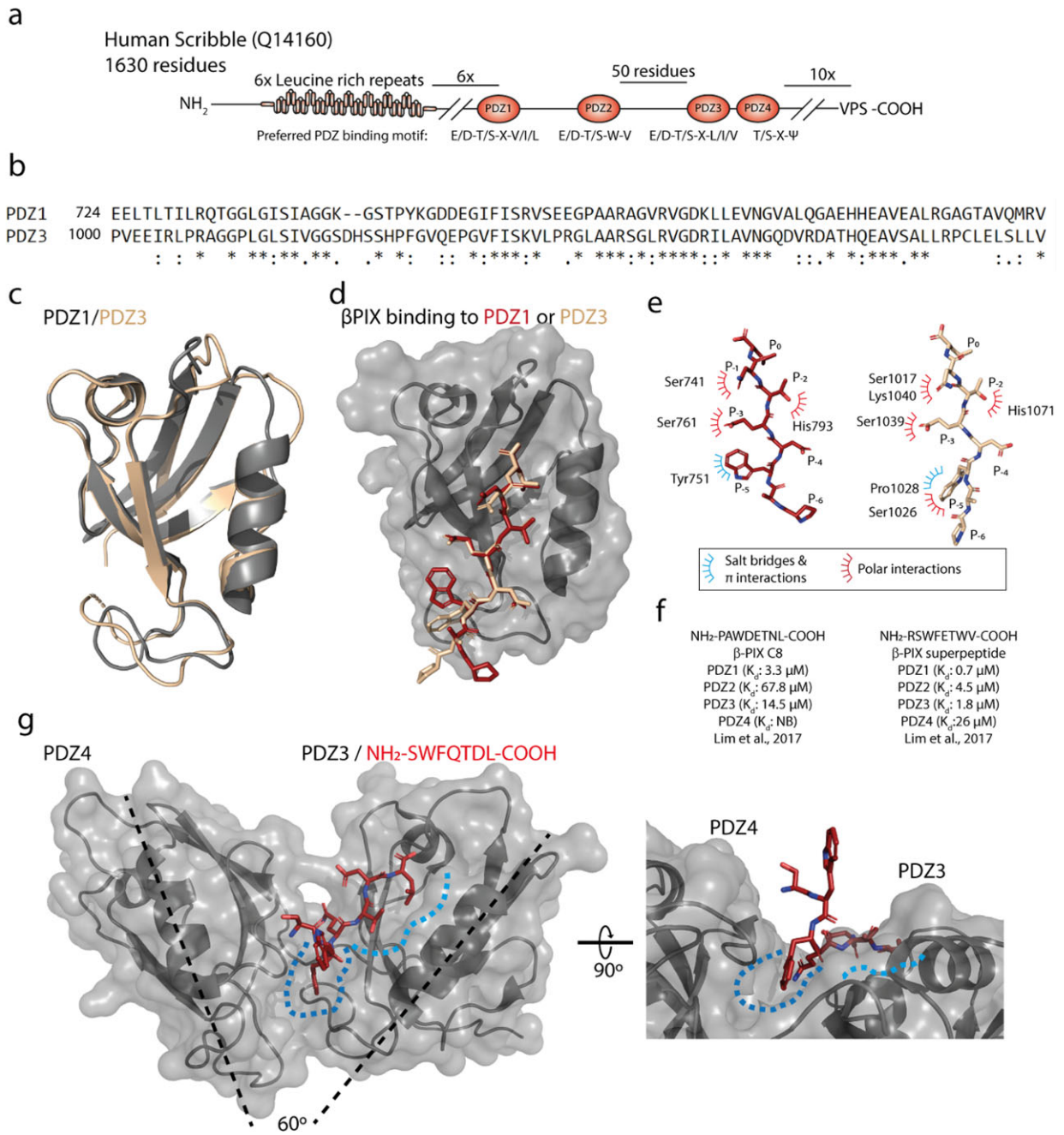


Figure 10. a) Domain organization of Scribble (Uniprot: Q14160) with PDZ ligand class preference of PDZ1-4 and Scribble C-terminal PDZ binding motif. b) Alignment (Clustal Omega) of Scribble PDZ1 and PDZ3 show about 40% full sequence conservation between PDZ1 and PDZ3. c) Structural overlay of scribble PDZ1 (PDB: 5VWK, grey) and PDZ3 (PDB: 5VWI, wheat) show high degree of structural conservation between the two domains. d) Structural overlay βPIX C8 peptide ($_{NH2}$-PAWDETNL$_{-COOH}$) (red: PDZ1 binding / blue: PDZ3 binding) with scribble PDZ1 (PDB: 5VWK, grey) show highly conserved backbone and side chain organization of βPIX P_0–P_{-4}, but less conserved main chain and side chain configuration of βPIX P_{-5}–P_{-7} when binding to PDZ1 or PDZ3. e) Side chain interactions of βPIX/PDZ1 and βPIX/PDZ3 are also highly conserved. The βPIX/PDZ1 interaction is stabilized by side chain interactions (red arcs) between P_{-1}, P_{-2}, and P_{-3} with Ser_{741}, His_{793}, Ser_{761}, respectively; furthermore, the interaction is stabilized by a CH–π interaction (blue arc) between P_{-5} Trp and Tyr_{751}. The βPIX/PDZ3 is stabilized by side chain interactions (red arc) between P_{-1}, P_{-2}, P_{-3}, and P_{-5} with Ser_{1017}/$Lys1040$, His_{1071}, Ser_{1039}, and Ser_{1026}, respectively; furthermore, the interaction is stabilized by a C_αH–π interaction (blue arc) between P_{-5} Trp and Pro_{1028}. f,g) Structure of the class II ligand $_{NH2}$-SWFQTDL$_{-COOH}$ binding to the PDZ3 of the PDZ3-4 tandem of Scribble (PDB: 4WYU). The structure shows a secondary binding pocket (indicated by dark blue dashed line) besides the canonical (indicated by light blue dashed line) where P_{-4} Phe inserts into a hydrophobic patch between PDZ3 and PDZ4 and P_{-3} Gln is coordinated by two Ser residues in PDZ3 and PDZ4, respectively (not shown).

packing against Tyr751, whereas in PDZ3, the Trp_{-5} is rotated and forms a hydrogen bond interaction with Ser1026, while also forming a $C_{\beta}H{:}\pi$ interaction with Pro1028. Sequence alignment of all four Scribble proteins reveal that the selectivity profile of β-PIX can be attributed to sequence differences rather than structural differences (Figure 10b,c).[239] The preference and selectivity for PDZ1 and PDZ3 over PDZ2 and PDZ4 was validated in a cellular context using endogenously expressed β-PIX. Further insight into the PDZ3-4 tandem showed that binding to PDZ3 of the class I ligand was enhanced by a secondary binding pocket forming between the canonical binding pocket in PDZ3 and the back side of the binding pocket of PDZ4 (Figure 10g).[240]

Phage display has been used to generate high-affinity peptide inhibitors targeting Scribble (Figure 10f).[241] One of the most promising hits, RSWFETWV, was shown to bind to PDZ1, PDZ2, and PDZ3 with IC_{50} values of 2.2, 4.7, and 3.9 μM, respectively.[241] The importance of the amino acid side chains in position 0 and −2 was confirmed by Ala substitutions. The affinity of this peptide was verified by SPR in an independent study and, as expected from previously reported results, the peptide is not selective and thus binds to PDZ1, PDZ2, PDZ3, and PDZ4 with K_d values of 0.7, 4.5, 1.8, and 26 μM, respectively.[239] Scribble is a promising target for the development of anticancer drugs; however, a major challenge is to design and develop PDZ selective inhibitors, which has so far not been achieved.

4.2.4. Targeting the PDZ Domains of Syntenin

Syntenin was described and characterized in 1996 as a novel melanoma differentiation-associated gene[242] and was hence named melanoma differentiation-associated gene 9 (MDA-9). Since then, several additional names including syntenin-1, syndecan binding protein 1 (SDCBP1), scaffold protein Pdp1, and pro-TGF-α cytoplasmic domain-interacting protein 18 (TACIP18) have been employed. Syntenin has been shown to regulate transmembrane-receptor trafficking, tumor-cell metastasis, exosome production, and neuronal-synapse formation.[243]

The syntenin gene (*SDCBP1*) is located on chromosome 8 [8q12.1] and encodes for a 33 kDa protein (298 aa), comprising a 113 aa unstructured N-terminal domain (NTD) followed by two adjacent PDZ domains and a short 24 aa C-terminal domain (CTD) (**Figure 11**a).[243] Sequence analysis across species has shown that syntenin is highly conserved with homologues in rat, mouse, zebrafish, and *Xenopus*.[244] Loss of syntenin function in mice shows no major effects, with only a mild phenotype in intestinal homeostasis and auditory-cued fear memory reported.[245,246] Syntenin is highly expressed during human fetal development and with relative low levels in adult tissue,[247] but a number of independent studies indicate that syntenin is overexpressed in various cancers, including melanoma, glioblastoma, breast cancer, and urothelial cell cancer.[244]

The scaffolding activity of syntenin is mainly regulated by the two PDZ domains (Figure 11a,b). Several NMR and X-ray crystal structures of ligands bound to syntenin reveal that interaction selectivity of the PDZ2 domain is determined by a combination of three binding pockets that bind amino acid side chains with different properties (Figure 11c,d,f). For example, syndecan-4 interacts with PDZ2 via P_{-1} and P_{-2} (Figure 11e), whereas the interleukin-5 receptor α (IL-5Rα) utilizes P_0 and P_{-1} (Figure 11f). Structural analysis of PDZ2 bound to various peptide ligands reveals that the binding site can induce conformational changes in the α2 helix, and thus adopt an induced fit, depending on the specific interaction partner.[243] Although most interaction partners of syntenin show a preference for PDZ2, Frizzled 7 (Fz7), insulin-like growth factor-1 receptor (IGF1R), and IL-5Rα have been described to have a higher affinity for PDZ1.[36,248,249] The PDZ domains of syntenin have also been found to interact with membrane lipids. In particular, phosphatidylinositol 4,5-bisphosphate (PIP_2) interacts with PDZ2. This interaction is also able to recruit the C-terminal of Fz7, forming a tridentate complex (Figure 11g).[55] As PIP2 is not present in some cellular compartments, this would favor specific cellular locations of syntenin.[243]

Syntenin has a unique function of facilitating both intracellular and extracellular events of invasion and has thus evolved as a promising target for cancer intervention. The first small-molecule syntenin inhibitor, 113B7 (Figure 11h), was developed by fragment-based drug design by NMR screening of an in-house assembled fragment library comprising about 5000 compounds against syntenin PDZ12.[250] Two fragments were identified to mainly interact with the PDZ1 domain and within the interface between the two PDZ domains. A combination of molecular docking and SAR studies guided the synthesis of the dimeric compound 113B7. The affinity was determined to $K_d = 21$ μM by NMR and shown to inhibit syntenin binding to EGFRvIII. Treatment with 113B7 in an in vivo glioma model resulted in smaller, less invasive tumors and enhanced survival.[250]

Phage display has been used to identify both internal and C-terminal peptide inhibitors of syntenin including more than 300 potential ligands.[251] The best-characterized phage display hit is the heptamer SKKEWYV peptide (Figure 11h). This sequence corresponds to the C-terminal of nectin-1, a protein involved in cell adhesion. SKKEWYV binds to full-length syntenin with a K_d of 14 μM. The interaction between syntenin and nectin-1 was further validated by immunoblots and co-localization of endogenous proteins in MCF-7.[251]

4.3. Targeting PDZ Domain Proteins Involved in Viral Infections

The first PDZ binding motifs in viral proteins were discovered just over 20 years ago in gene products found in human T-lymphotropic virus type 1 (HTLV-1), human papilloma virus (HPV), and human adenovirus (Ad).[252–254] HTLV-1 is a human retrovirus, which infects CD4+ T lymphocytes. The HTLV-1 transcription factor Tax is a crucial element in malignant transformation of infected cells and it contains a C-terminal PDZ binding motif.[255,256] Interestingly, the Tax protein in the closely related and non-leukemogenic HTLV-2 lacks this PDZ binding motif and has a significantly lower transformation potential than HTLV-1 Tax.[257] The human adenovirus type 9 (Ad9) E4 region ORF1 gene product, E4-ORF1, is another viral protein, which targets several PDZ-containing proteins through a C-terminal PDZ binding motif. Ad9 causes eye infections in humans but, in other mammalian species, the viral E4-ORF1 plays a key role in tumorigenesis.[258]

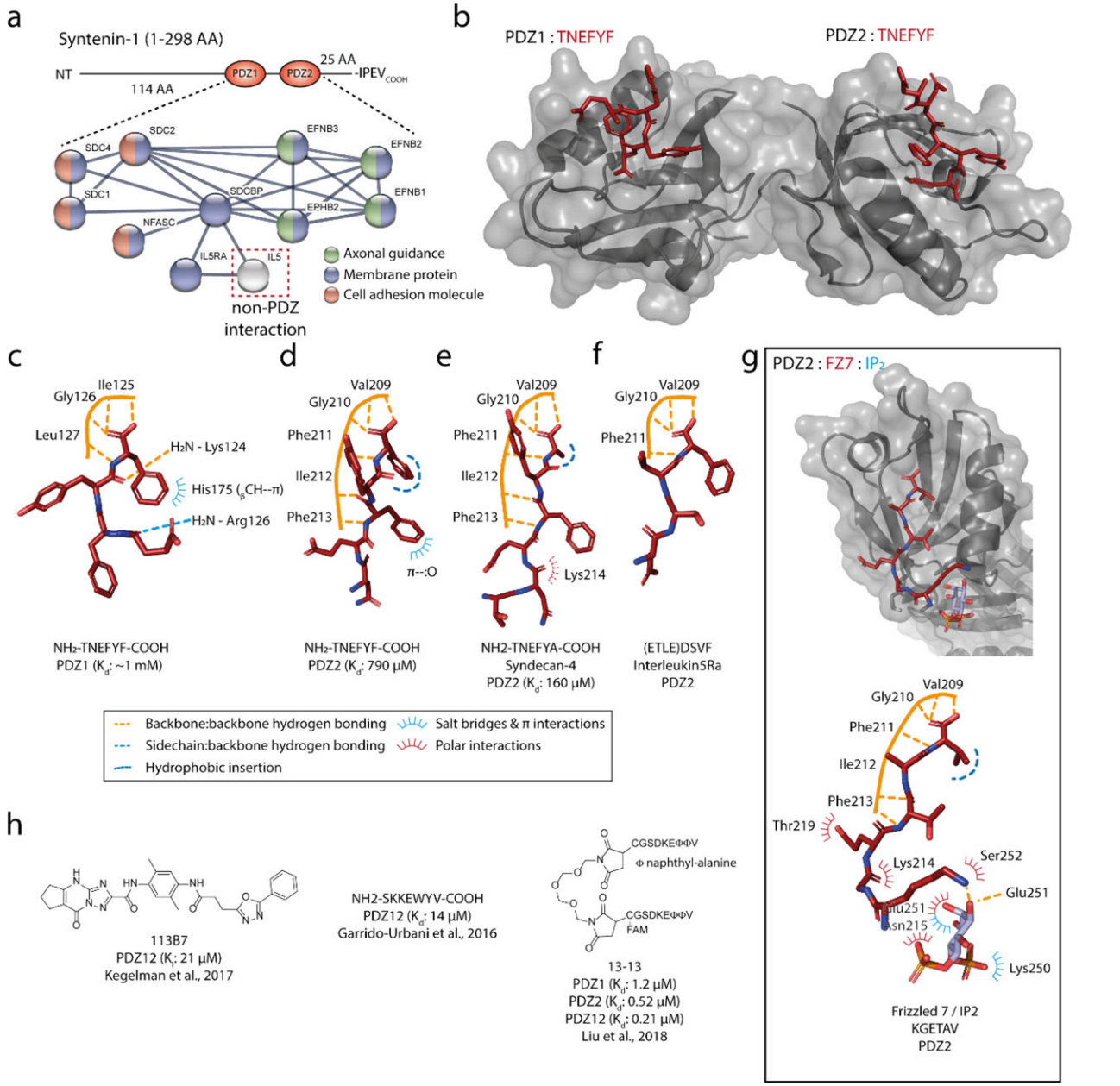


Figure 11. a) Domain organization of Syntenin (Uniprot: O00560) and protein interaction network (STRING) showing the highest confidence proteins interacting with Syntenin PDZ domains, most of which are transmembrane proteins. b) Structure of Syntenin PDZ1-2, dual occupied by a syndecan-derived peptide ($_{NH2}$-TNEFYF-$_{COOH}$), shows perpendicular orientation of the PDZ1 and PDZ2 binding pocket (PDB: 1W9E) and noncanonical insertion of the peptide in PDZ1. c) Hydrogen bonding network of $_{NH2}$-TNEFYF-$_{COOH}$ binding to Syntenin PDZ1 shows a noncanonical insertion of the peptide. The C-terminal carboxylic acid is stabilized by hydrogen bonding to the Ile125-Gly126-Leu127 loop backbone, while the P0 (Phe) sidechain interacts with His124 through a $C_\beta H-\pi$ interaction and the P_{-1} backbone carbonyl interacts with Lys124 sidechain amine; the P_{-3} (Glu) backbone amine coordinates an Arg126 sidechain amine. d) Hydrogen bonding network of $_{NH2}$-TNEFYF-$_{COOH}$ binding to Syntenin PDZ2 shows canonical insertion of the peptide, with hydrogen bonding of P_0 and P_{-2} backbone carbonyls/amines to βB and hydrophobic insertion of P_0 (Phe) side chain into the hydrophobic pocket. e) Hydrogen bonding network of the endogenous Syntenin PDZ2 ligand, Syndecan-4, shows almost identical hydrogen bonding network as (d), but with the P_{-4} carbonyl partially coordinating (red dashed arc) the side chain amine of Lys_{214}. f) The binding of interleukin5Ra C8 ($_{NH2}$-ETLEDSVF-$_{COOH}$) relies on a canonical binding of the C-terminal carboxylic acid, but otherwise binds as a noncanonical ligand, almost perpendicular to the binding pocket. g) Structure (top) and hydrogen bonding network (bottom) of Frizzled7 C6 ($_{NH2}$-KGETAV-$_{COOH}$) and lipid derived Inositol 1,3-bisphosphate (IP_2) coorporating in the binding to Syntenin PDZ2. The hydrogen bonding network shows normal backbone interactions between the peptide and the domain, and is tightened by side chain interaction between P_{-3} (Glu) and P_{-5} (Lys) and Thr_{219} and Ser_{252}, respectively. The interaction is stabilized further by interactions between P_{-4} Gly carbonyl coordinating Lys214 and P_{-5} Lys side chain also coordinating a hydroxide H from IP_2. The interaction between PDZ2 and IP2 is coordinated by several hydroxide H bonds to the side chain (red arc) and main chain (red arc) of the PDZ domain. h) Structures of inhibitors targeting Syntenin PDZ domains.

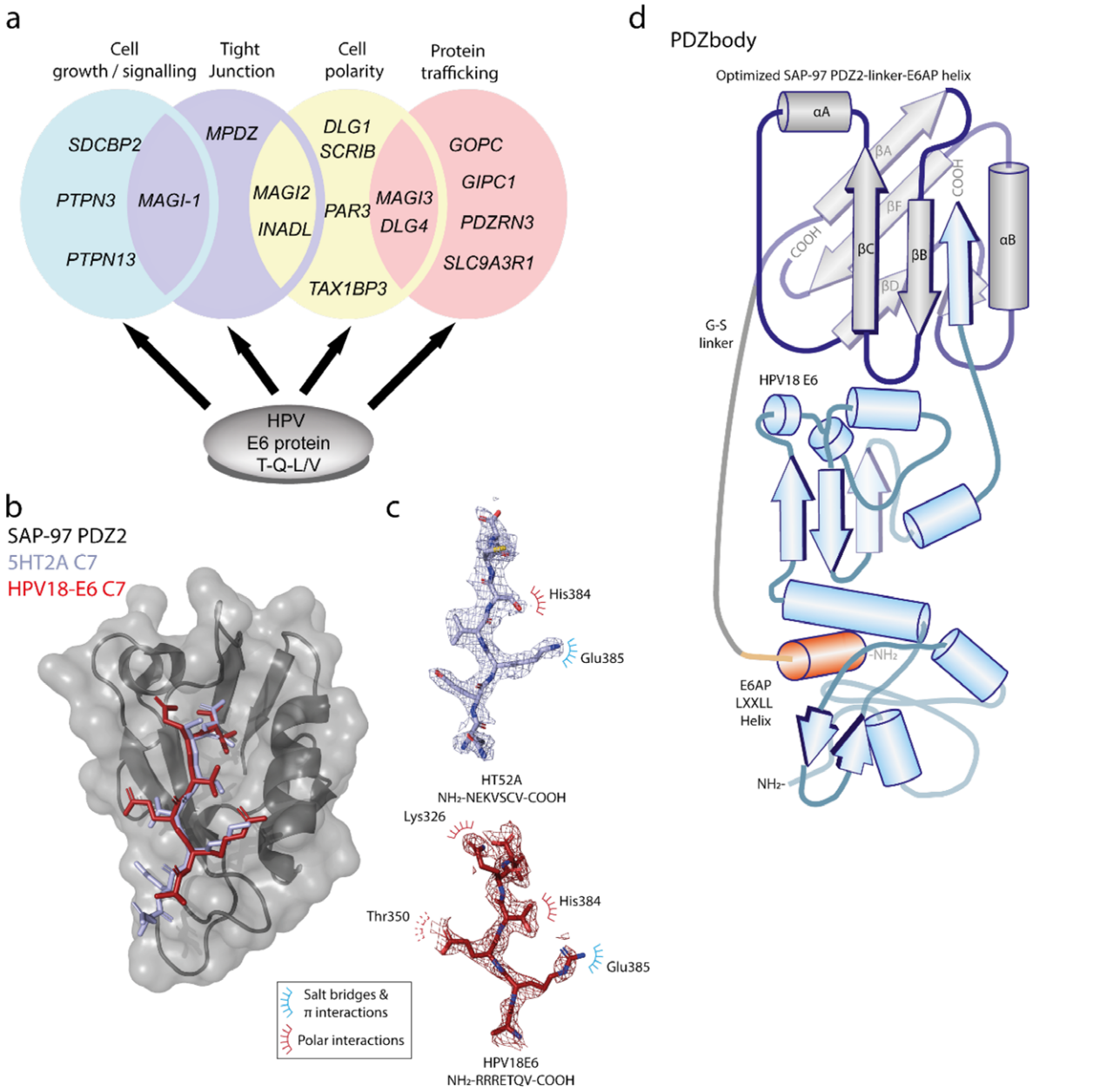


Figure 12. a) PDZ-interaction partners of the HPV18-E6 protein and their respective cellular involvement. b) Structure of SAP-97 PDZ2 with overlay of the endogenous ligand 5HT2A C7 ($_{NH2}$-NEKVSCV-$_{COOH}$, PDB: 4OAJ) (red) and the viral HPV18-E6 C7 peptide ($_{NH2}$-RRRETQV-$_{COOH}$, PDB: 2I0L) which show very similar insertion of the two peptides into the binding pocket. c) Electron density map (2Fo-Fc) of the peptides shows very similar electron density distribution P_0–P_{-4}, despite low sequence similarity. Especially the noncanonical interaction between P_{-4} (Lys) (5HT2A) and Glu_{385} and P_{-4} (Arg) (HPV18-E6) and Glu_{385} shows similar electron density suggesting targeted evolution of the HPV18-E6 toward mimicking endogenous PDZ ligands. d) Graphical model of the PDZbody[259,275] where the HPV18-E6 protein binds to the canonical binding pocket in the optimized SAP-97- PDZ2 through its C-terminal residues. To obtain additional affinity and specificity, the optimized SAP-97 PDZ domain was fused to an α-helix from E6-associated protein (E6AP) through a Gly-Ser linker, making up the PDZbody[259] or PDZ-LxxLL chimera.[275]

Human Papillomavirus (HPV) E6 and E7 hijack the cell during infection by interacting with endogenous proteins such as the well-described transcription factor and tumor suppressor, p53, and several PDZ-containing proteins.[259] By binding to the E6AP ubiquitin ligase, HPV E6 targets p53 and the PDZ domain proteins for degradation (**Figure 12**a).[253,260] Close to 200 different types of HPV have been documented, but only a small portion of these may cause cancer; the so-called high-risk HPVs. While HPV protein E6 has been shown to interact with p53 and E6AP in both low and high-risk HPVs,[260,261] only the high-risk cancer-causing HPV E6 proteins contain a C-terminal PDZ binding motif, which is recognized by different PDZ domain proteins depending on the HPV strain.[262] High-risk HPV E6 proteins have been shown to associate with a plethora of

PDZ domain proteins including cell polarity proteins and tight junction proteins as well as trafficking and signalling proteins (for an extensive review, see ref. [255]).

PDZ binding motifs and interactions with PDZ domain proteins have also been reported for several other viruses such as hepatitis B virus, whose core protein has a noncanonical C-terminal PDZ binding motif that has been shown to interact with the PDZ domains of PDZ domain-containing protein GIPC1 (GIPC1)[263] and protein-tyrosine phosphatase PTPN3.[264] In severe acute respiratory syndrome–related coronavirus, PDZ binding motifs have been found in viral proteins, 3a and E, which play key roles in viral entry, release, and genome replication. Mutational analysis has shown that the presence of at least one functional PDZ binding motif in either 3a or E is necessary for virus viability.[265] In Kaposi's sarcoma herpesvirus, expression of the PDZ-LIM domain-containing protein 2 gene (*PDLIM2*) is repressed, which enables tumorigenesis and tumor maintenance in infected cells.[266] In HIV-1, syntenin is recruited to the plasma membrane in infected cells, and overexpression of syntenin has been shown to reduce HIV-1 infectivity.[267] A wide variety of PDZ domain proteins have been found to interact with viral proteins. However, a few PDZ domain proteins seem to be more common than others. This subset of cellular PDZ domain proteins is targeted by otherwise unrelated viral proteins, which demonstrates the importance of understanding these specific binding events and their cellular effect in the quest for developing new antiviral drugs.

4.3.1. Targeting the PDZ Domains of SAP-97 and Scribble

Synapse-associated protein 97 (SAP-97) is transcribed from the *DLG1* gene, which is widely expressed in humans and other vertebrates. It is, as previously described, part of the MAGUK family (Figure S3, Supporting Information).[268] SAP-97 plays a key role in regulating apico-basal polarity of epithelial cells as part of the highly conserved Scribble-Dlg-Lgl complex. Scribble is involved in the regulation of cell polarity, cell shape, adhesion, and several signaling pathways,[232,233] as previously described. Both SAP-97 and Scribble have been shown to interact with HTLV-1 Tax PDZ binding motif, which affects their function and subcellular localizations, removing them from the cell membrane.[254,269] Overexpression of HTLV-1 Tax has also been shown to inhibit the ability of SAP-97 to arrest cell cycle progression.[270]

The HTLV-1 Envelope glycoprotein (Env) has also been shown to interact with SAP-97 during viral infection. HTLV-1 Env contains a canonical C-terminal PDZ binding motif, which is conserved in HTLV-2. However, a mutant virus lacking this motif has been found to be unable to recruit SAP-97, with a consequent reduced ability to transmit infection, which in non-mutant HTLVs occurs by triggering cell–cell contact between T lymphocytes.[271] While SAP-97 acts as a tumor suppressor in the case of HTLV-1, Ad9 E4-ORF1 protein is suspected of exerting a gain-of-function upon SAP-97, which disrupts its function in the tight junction and relocalizes to the plasma membrane, where it mediates phosphatidylinositol 3-kinase (PI3K) activation.[272] This activation of PI3K deregulates cytosolic signalling pathway, which leads to cell survival and metabolism, and enhances virus replication.[273]

HPV E6 binds to and targets both SAP-97 (Figure 12b, c) and Scribble for proteasomal degradation through its C-terminal PDZ binding motif.[274] Because of the role HPV E6 plays in transforming infected cells into cancer cells, it is considered a potential drug target. HPV E6 binds E6AP and the PDZ domain proteins through two different epitopes; E6 contains a class I PDZ binding motif, while it recognizes a LxxLL-motif in an α helix of E6AP. This was exploited in the generation of a double-epitope inhibitor by cloning and expressing an optimized SAP-97 PDZ domain linked to a motif-carrying α helix of E6AP, both of which bind to the E6 protein at different sites. The measured K_d of this bivalent binder, denoted PDZbody, was greatly improved compared to wild-type PDZ binding affinities (Figure 12d).[259] A similar approach was employed by another group, who instead modelled the PDZ part of the double-epitope inhibitor after the MAGI1 PDZ2.[275] In addition to the more well-studied interactions with viral proteins from HTLV-1, Ad9, and HPV, SAP-97 and Scribble have also been associated with Hepatitis C virus. In infected cells, SAP-97 expression has been shown to decrease, while Scribble co-localized with the Hepatitis C core protein.[276] In HIV-1, SAP-97 has been shown to co-localize with the HIV-1 Gag protein and restrict infectivity.[277]

4.3.2. Involvement of Tight Junction PDZ Domain Proteins in Viral Infections

The tight junctions are prevalent entryways for several viruses, both through the integral membrane proteins and through the cytoplasmic proteins associated with tight junctions; of which many are PDZ domains.[255,258] Besides SAP-97 and Scribble, the HPV E6 protein is associated with the tight junction PDZ domain proteins MAGI1, MUPP1, and Par3 (Figure 12a). Interaction with HPV type 18 E6 protein promotes a relocalization of the polarity protein, Par3,[278] whereas both MAGI1, a MAGUK protein related to SAP-97, and the multi-PDZ domain protein, MUPP1, are targeted for degradation.[279,280] MAGI1 and MUPP1 are also the targets of Ad9 E4-ORF1 protein, which relocates the PDZ domain proteins to the cytoplasm, contributing to viral transformation.[279,280] Similarly, Ad9 E4-ORF1 also binds to another MAGUK family protein, ZO-2, and sequesters it in the cytoplasm. In contrast to this, the non-tumorigenic Ad type 5 and type 12 are unable to bind ZO-2.[281] The ZO-2 homolog, ZO-1, which plays an important role in tight junctions, has been associated with the HIV-1 glycoprotein 120. Though no PDZ binding motif was found in the HIV-1 protein, cells exposed to the glycoprotein showed a mislocalization of ZO-1 from the tight junction, followed by a decreased expression level.[282]

4.4. PDZ Domain Protein Targets in Cystic Fibrosis

The cystic fibrosis transmembrane conductance regulator (CFTR) associated ligand (CAL, also known as GOPC, FIG, and PIST) gene *(GOPC)* is placed on chromosome 6q22.1, and is widely expressed in several different tissues (NCBI gene: 57120). Inactivation of the *GOPC* gene has shown to trigger globozoospermia in mice.[283] Gene variants and mutations

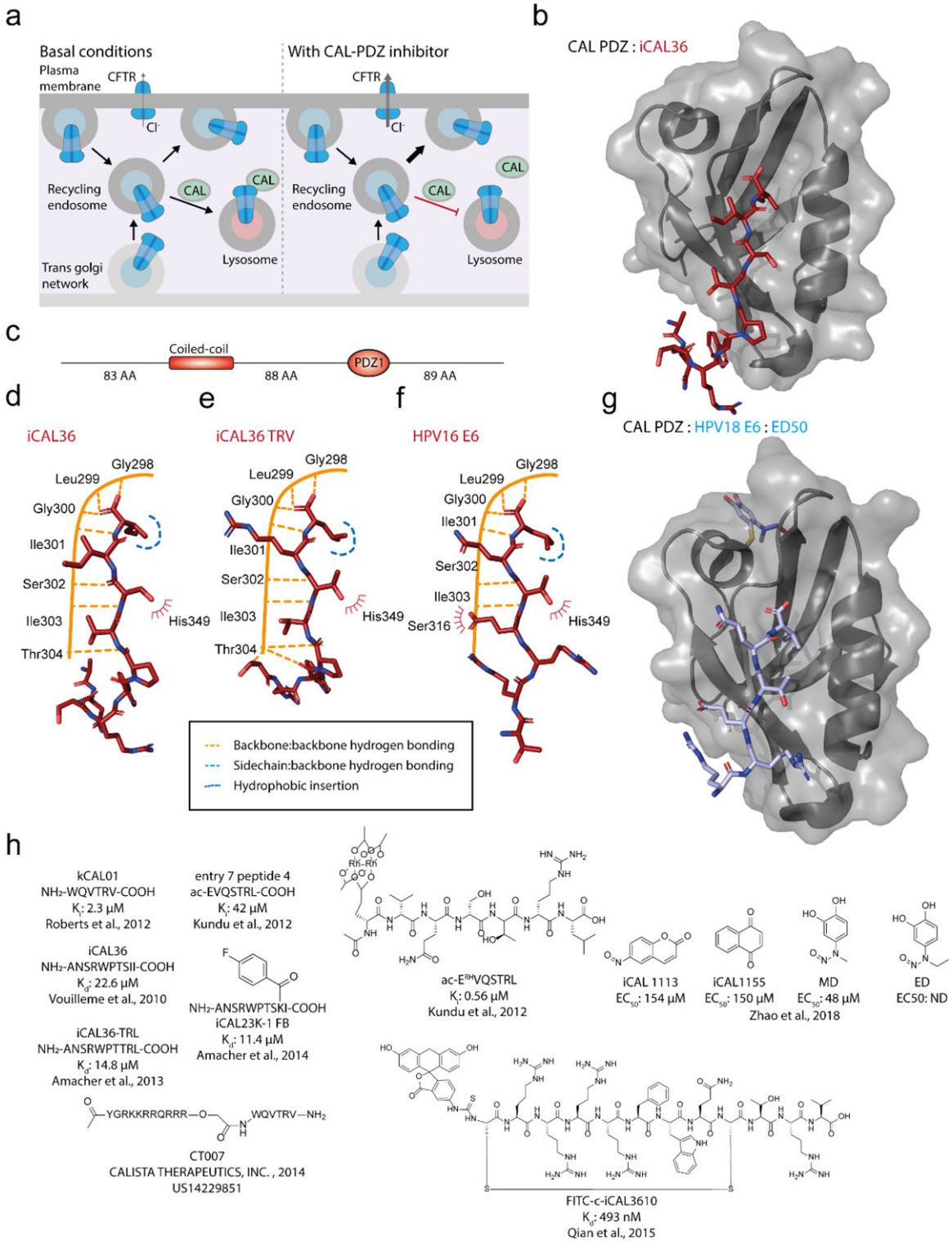


Figure 13. a) Under normal conditions, CAL is involved in trafficking of CFTR to the lysosomes, which in some branches of the disease cystical fibrosis (CF) enhances the pathological features of the disease (Δ508-CFTR). Inhibitors targeting the interaction between CFTR and CAL through the PDZ domain of CAL have been suggested as supplementary treatment for CF, through inhibition of CFTR degradation. b) Structure of the PDZ domain of CAL in complex with the inhibitory peptide iCAL36. c) Domain structure organization in CAL (Uniprot: Q9HD26) shows a coiled-coil domain and a PDZ domain, both flanked by unstructured regions. d–f) Hydrogen bonding network shows canonical insertion of the class I ligand; d) iCAL36 ($_{NH2}$-ANSRWPTSII.$_{COOH}$), e) iCAL36-TRV ($_{NH2}$.ANSRWPTTRV.$_{COOH}$), and f) HPE16 E6 ($_{NH2}$-RRRETQV.$_{COOH}$) with hydrogen bonding between the ligands and the PDZ backbone, insertion of P0 in the hydrophobic pocket, and coordination of the P_{-2} side chain to the αB1 His349. h) Structure of the currently known CAL PDZ inhibitors.

of the PDZ domain of CAL has been found in several cancer types (Table S3, Supporting Information). Gene rearrangements between the *GOPC* gene and *ROS1* gene have been found in patients with lung adenocarcinomas[284] where osimertinib resistance is seen.[285] The emerging role of CAL is trafficking of several membrane proteins between intracellular compartments, primarily between the trans Golgi network (TGN), endosomes, and lysosomes, thus reducing receptor surface expression and promoting protein degradation (**Figure 13**a).[286–288]

In humans, CAL has been found to interact with several different proteins, including ion channels, GluD2 and CFTR, GPCRs including the β1 adrenergic receptor, and the trafficking protein, Syntaxin 6.[77,289] In particular, the interaction between CFTR and CAL has been studied due to its role in cystic fibrosis, where CAL promotes degradation of CFTR in lysozymes, and thereby reduces the amount of CFTR expressed at the surface (Figure 13a).[290–292] CAL is expressed as a 461 aa protein (Uniprot: Q9HD26), containing an unstructured N terminal region, a coiled-coil domain, a PDZ domain, and a C-terminal unstructured region, in many ways similar to PICK1, also containing unstructured N- and C-termini, a PDZ domain, and a multimerization domain (Figure 13c). Three different isoforms resulting from alternative splicing have been found; isoform 2 lacks 7 residues in the coiled-coil domains, potentially affecting the homo and hetero-multimerization. Isoform 3 is truncated after position 319, and the remaining C terminal residues are substituted changing the C-terminal to a PDZ binding motif (Tyr-Leu-Val).

The PDZ domain of CAL contains a conserved His at the N-terminal of αB, indicative of a class I ligand preference (S/T-X-Φ). Currently, 28 structures have been deposited in PDB, all showing the regular PDZ domain topology, with the primary difference in the βB-βC loop that in most cases is annotated with a partial helical structure, indicating a less flexible loop compared to other PDZ domains (PDB: 5IC3, 5K4F, 4Q6S). Interestingly, in most deposited structures, an aromatic residue (Trp, Phe, or Tyr) at position P_{-5} has a stacking interaction with His309, a feature that seems to act as a non-motif specificity residue (Figure 13b,d).[293] Mutating this residue had a profound effect on affinity, resulting in a dramatic loss of affinity highlighting the importance of this upstream interaction.[293] To engineer selective PDZ domain inhibitors, 6223 known PDZ domain ligands were screened (11-mer peptides)[294] using inverted peptide SPOT arrays[295] against five different PDZ domains, selecting 80 sequences for further examination.[296] From these 80 peptides, sequence alignment defined a consensus of S/T-x-L for peptides binding to CAL. A subset of the 80 peptides was further screened using a positional scan from P_0 to P_{-10}, with 20 different amino acids obtaining the C-terminal core peptide (P_0–P_{-3}). The optimized peptide was then combined with an N-terminal elongation library screening for P_{-4} and P_{-5} position selectivity. The peptide WPTSII resulting from this approach had a modest affinity of 33 μM. However, through optimizing the peptide for CAL, it lost affinity for the four other tested PDZ domains also interacting with CFTR, resulting in a 150-fold increase in selectivity. Elongating the peptide further to ten residues resulted in the final peptide, $iCAL36_{10}$, with an affinity of 22.6 μM, which maintained selectivity for CAL (Figure 13d,h).[296] Later, iCAL3610 was shown to be a competitive inhibitor of CFTR binding to CAL, which stabilized the dF508-CFTR variant surface expression and thereby increasing CFTR conductance.[297]

Additional work on iCAL3610 showed that substituting P_{-1} Arg with a Lys coupled to fluorobenzene at the side chain amine increased the affinity twofold (Figure 13h).[298] Substitution of P_0–P_{-2}, from Ser-Ile-Ile to Thr-Arg-Val ($iCAL36_{TRV}$), was also shown to yield a modest increase in affinity (Figure 13e,h). To make the peptide kCAL01[299] (WQVTRV) cell permeable and more plasma stable,[300] the P_{-3} Val was substituted to Cys and fused to a CPP motif (CRRRRF) that, upon oxidation, formed an intramolecular di-sulfide bridge between P_{-3} Cys and the N-terminal Cys (Figure 13h).[300] The peptide was found to bind to CAL with a K_d of 493 nM under reducing conditions, while not binding to CAL under nonreducing conditions. Furthermore, it was shown that cyclization increased the serum half-life and cellular uptake compared to linear and non-CPP peptides, respectively.[300] Based on the core motif of iCAL3610 and $iCAL3610_{TRV}$, Calista Therapeutics developed a series of inhibitors combining the iCAL36 core motif and a Tat CPP moiety connected by different linkers.[301] The compounds (CT003 and CT004) had no significant additive effect on the CFTR conductance when administered together with VX809 (Lumacaftor). Acetylation of the N-terminal, insertion of an esterase cleavable linker, and C-terminal amidation (Ac-YGRKKRRQRRR-Glycoyl-WQVTRV-NH_2, Figure 13h) was found to enhance the effect of VX809, when measuring the channel conductance. Nevertheless, it remains unclear if the N-terminal amidation still allows for binding to the PDZ domain of CAL.[301]

Recent work has shown that PDZ domain inhibitors of CAL can also be noncompetitive inhibitors and work through allosteric modulation of the canonical ligand binding.[93] Screening of a small-molecule library consisting of 5600 compounds, identified three compounds, iCAL1155, iCAL1113, and MD,[93] which all bound to the PDZ domain of CAL with affinities of 48–154 μM (Figure 13h). While iCAL1155 and iCAL1113 primarily perturbed residues in and around the canonical binding pocket, MD was shown to perturb an area adjacent to the binding pocket, which was also confirmed by the X-ray crystal structure (Figure 13h). Here, the Cys side chain is covalently linked to MD, but this linkage was highly dependent on experimental conditions. In the case of iCAL1113 and iCAL1155, both compounds were found to be highly cytotoxic, affecting both cell proliferation and inducing cell apoptosis.[93] In another study, using of cysteine-reactive compounds ED (Figure 13h) and EM was found to modulate the binding of HPV16 E6 to the PDZ domain of CAL (Figure 13f–h). Although the ED, EM, and MD most likely have limited therapeutic use, targeting PDZ domains with allosteric inhibitors through reactive Cys residues might have other uses. Comparison of all PDZ domain sequences shows that 162 out of 267 PDZ domains contain one or more Cys in their sequence (Table S1, Supporting Information). An example is the RGS3 (regulator of G protein signaling 3), which contains four Cys residues in its PDZ domains, three positioned in the bottom of the canonical binding pocket, potentially acting as a binding pocket gating mechanism (PDB: 2F5Y). The discovery that CAL is a key influencer of CFTR channel degradation and current modulator in bronchial epithelial cells led to the idea of targeting CAL for the treatment of cystic fibrosis in patients identified with the dF508 variant, increasing the surface lifetime

of the channel and restoring channel conductance. However, high affinity and selective inhibitors of CAL are still to be developed.

5. Potential Future PDZ Domain Protein Drug Targets

Modern "omics" studies, combined with interaction arrays and peptide libraries, have been a key resource for mining new drug targets for human diseases.[302] One of the first clues for potential targets comes from tissue expression profiles and how this expression is changed in disease states. Additionally, the potential target must be validated to demonstrate a direct link between the pharmacological intervention and the modification of the disease mechanism.

PDZ-containing proteins are distributed mainly in tissues that demand cell polarity to exert their function.[303] The human protein atlas pointed to the cerebral cortex and the smooth muscle as tissues with the highest transcript counts of PDZ-containing proteins, that may reflect the directional requirement of synaptic transmission and muscle contraction, respectively (**Figure 14**).[147] Even though many PPIs in the muscles are mediated through PDZ domains, such as the α-syntrophin–nNOS interaction,[304] their potential as pharmacological intervention points have not yet been suggested. In the brain, two PDZ-containing proteins have emerged with potential applications in Alzheimer's disease (AD): the Munc-18 interacting proteins (Mints), or X11, and the high-temperature requirement serine protease A1 (HtrA1). Finally, epithelial tissues also require strict cell-polarity, and the partitioning-defective (Par) protein complex was suggested to be one point of intervention in cancer therapeutics.

The Mints are a family of intracellular adaptor proteins that includes the brain-specific Mint1 and Mint2 and the ubiquitously expressed Mint3.[305] Mints share a similar domain structure comprising a variable N-terminus and a more conserved C-terminus. The Mint1 N-terminus contains a Munc-18-interacting (MI) region followed by a calcium/calmodulin-dependent serine protease interacting (CI) region, while Mint2 contains only the MI region, and Mint3 has neither of the regions. The C-terminal region is conserved among all Mints and comprises a phosphotyrosine binding (PTB) domain, followed by an α-helical linker (ARM) and two tandem PDZ domains (**Figure 15**a).[306] The relevance of Mints in AD first arose from the co-localization of Mints with the amyloid deposits in postmortem human brain slices.[307] However, the effects of Mint2 knockout in mice models of AD has been inconsistent, resulting in either increased or decreased amyloid beta (Aβ) levels.[308,309] The molecular mechanism suggested for the role of Mints in AD involve the trafficking of the amyloid precursor protein (APP) to the γ-secretase complex promoted by PPIs between both Mint-APP and Mint-presenilin-1.[310] The PTB domain of Mint proteins interacts with APP C-terminal region peptide motif, YENPTY, while the first PDZ domain of Mint interacts with presenilin-1, a constituent of the γ-secretase complex.[311–315] A potential peptide inhibitor of the Mint(PTB)–APP or the Mint(PDZ)–presenilin interactions could reduce the Aβ levels by preventing the recruitment of APP to the γ-secretase complex. Further evidence for this mechanism comes from the reduction in the Aβ levels in mice brain after the genetic replacement of APP gene (*APP*) with a variant lacking the last 15 amino acids, including the YENPTY sequence.[316] In addition to presenilin and APP, some additional Mint interaction partners have been reported, which are also believed to play a role in the amyloidogenic pathway.[317]

Another protein potentially relevant in the treatment of AD is HtrA1. In humans, this protein belongs to a family of proteases comprising HtrA1-4, and is composed of a regulatory N-terminal region, followed by a serine-protease domain and a C-terminal PDZ domain (Figure 15b).[318] HtrA1 is widely expressed in human tissues, is enriched in the skin, reproductive tissues, and the brain, and is found both in the cytoplasm and in the extracellular space.[319] In the cortex of the human brain, it is the most expressed PDZ-containing protein, with almost twice the transcript-count of PSD-95.[147] Its expression is downregulated in some breast, brain, and liver cancers and it was described as a tumor-suppression factor promoting cell death.[320] The relevance of HtrA1 in AD was suggested first in 2005 due to its co-localization with Aβ in human brain slides using immunohistochemistry. Furthermore, the incubation of an HrA1 protease inhibitor dose-dependently reduced the Aβ levels in non-transfected astrocytes.[321] Later, it was demonstrated that HtrA1 could degrade aggregated tau protein, another critical feature in AD pathology, and that tau and HrA1 co-localize in human AD brain slices.[322] The role of PDZ domains in Aβ and tau was recently investigated. In bacterial HtrA, PDZ domains exert allosteric control of the proteolytic activity.[323] This contrasts with human HtrA1, which does not require PDZ domains for fibril cleavage and disaggregation of both tau and Aβ protein.[324] However, the length of cleavage products was influenced by the presence of the PDZ domain, suggesting that its precise function in substrate processing is still unclear. The discovery of PDZ ligands that can enhance the proteolytic activity of HtrA1, similarly to the bacterial HtrA, could represent a critical therapeutic intervention for AD.

In epithelial cells, the ability to define an apical and a basal compartment is an essential requirement to respond effectively to changes in the intra- and extracellular environment.[325] One of the major protein complexes in the cellular polarization process consists of Par3, Par6, and the atypical protein kinase C (aPKC).[326] Only Par3 and Par6 contain PDZ domains, which are C-terminally flanked by a Phox and Bem1 (PB1) domain in Par6, and a PB1-like homo-oligomerization domain in Par3. Par6 contains one PDZ domain, while Par3 has three (Figure 15c). Par3 and Par6 are expressed in low levels in normal human tissues, but its upregulation was described in renal carcinomas,[327] and in gastric cancer.[328] Par3 is essential for the delivery of PKC to the apical epithelial surface,[329] and the Par6 C-terminus can bind and localize Par3 in the membrane, through the PDZ domain of Par3.[330] As the Par complex plays a pivotal role in controlling cell polarity, it is not unexpected that they are also implicated in human carcinogenesis. For example, knockdown of Par3 with RNAi triggers a rapid tumor growth with metastatic colonization in the presence of oncogenes.[331] Targeting cell polarity signaling could represent a novel therapeutic intervention to block cell proliferation and prevent epithelial-to-mesenchymal transition (EMT). In particular, Par6 protein is involved in the TGF-β pathway in cancer cells.[332] The overexpression of the C-terminal S345A mutant

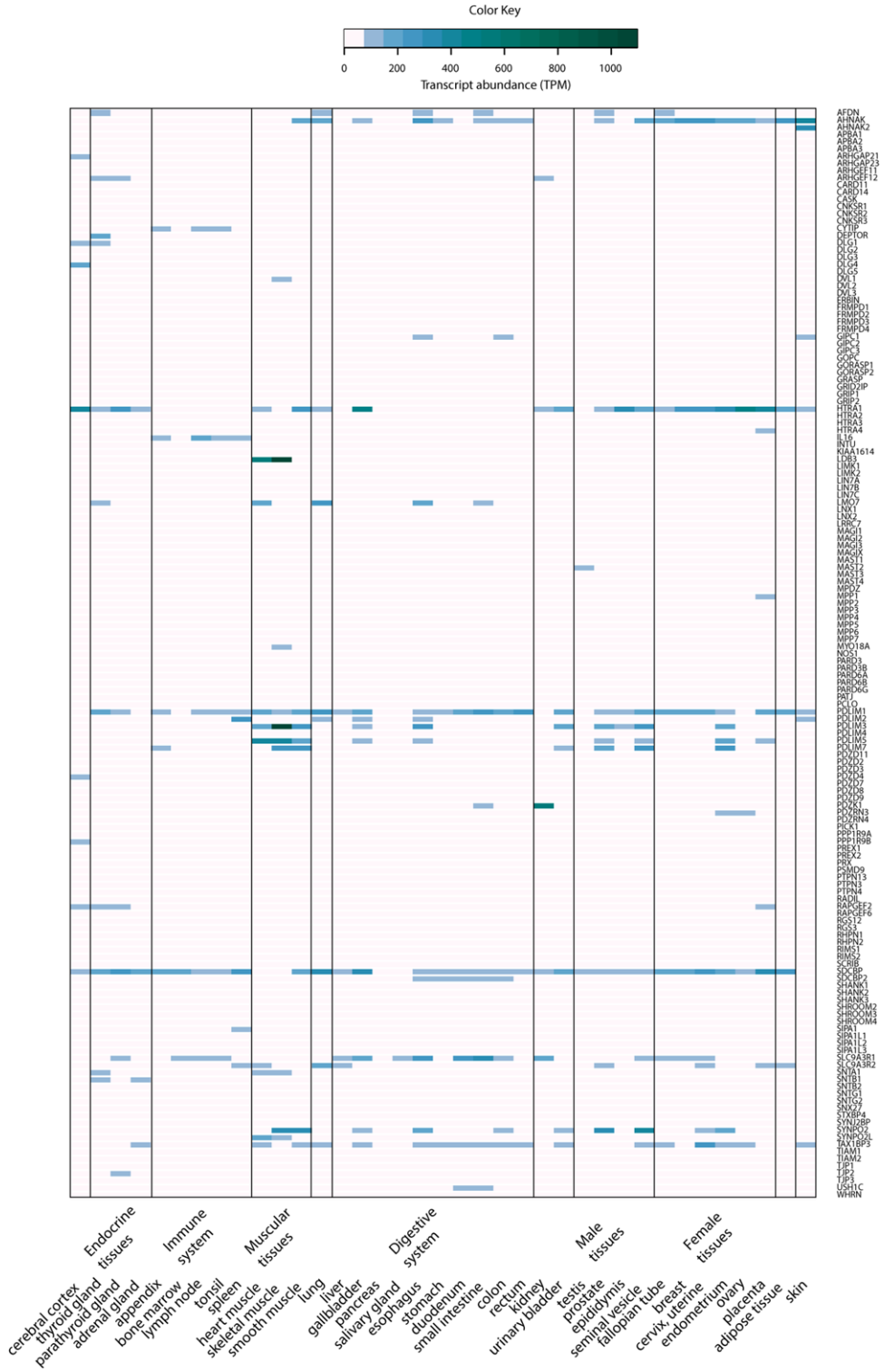


Figure 14. mRNA expression levels of 151 PDZ-containing proteins distributed in 37 human tissues according to the human protein atlas. The abundance in "Transcript Per Million" (TPM) is reported as the sum of the TPM values of all its protein-coding transcripts.

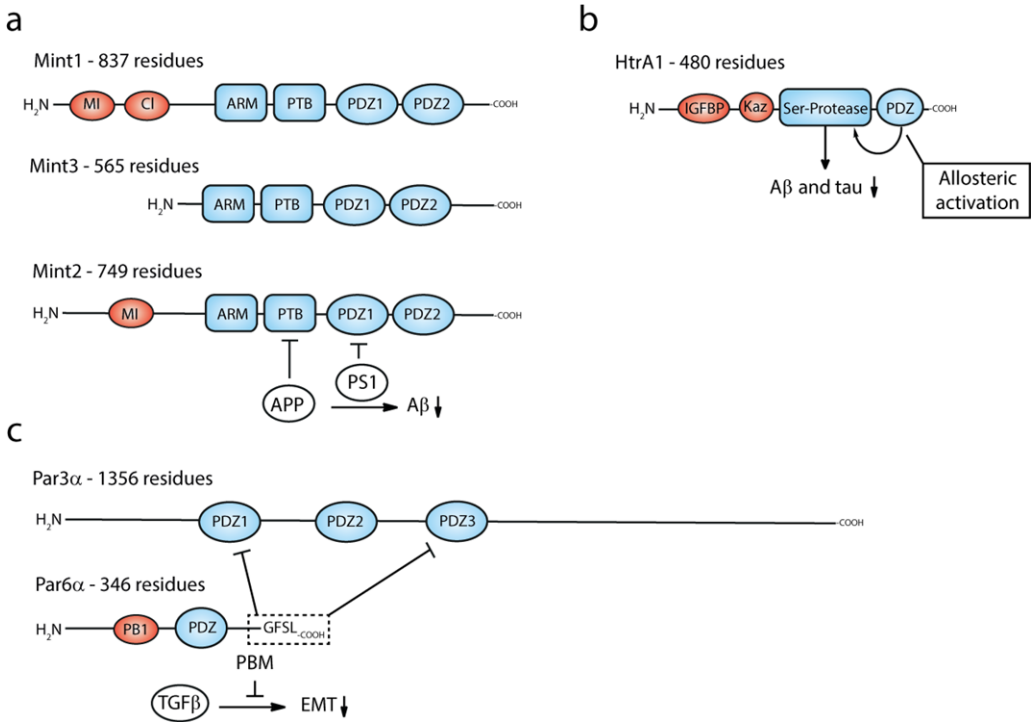


Figure 15. Domain organization of selected PDZ-containing proteins and potential therapeutical interventions. a) The Munc-18 interacting proteins 1-3 (Mint1-3). Inhibition of either the phosphotyrosine binding domain (PTB) or the PDZ domain protein–protein interactions (PPIs) could reduce amyloid precursor protein (APP) metabolism and Aβ generation. b) High-temperature requirement serine protease A1 (HtrA1). Binding to the PDZ domain of HtrA1 may induce allosteric activation of the serine protease domain (Ser-protease), that can disaggregate and cleave Aβ and tau proteins. c) The partitioning-defective proteins 3 and 6 (Par3 and Par6). Par6 C-terminal PDZ binding motif interacts with both PDZ1 and PDZ3 of Par3. Inhibition of Par6 PPIs may modulate the Par6-TGF-β signaling and reduce cancer metastasis through epithelial-mesenchymal transition (EMT).

of Par6 reduced the EMT in NmuMG cells, and also the number and incidence of metastatic lung tumors.[333] The modulation of the TGF-β-Par6 signaling could represent a selective intervention in cancer treatment.

6. Conclusion

PDZ domain–containing proteins are playing a plethora of important roles in cell signaling, particularly as mediators of signaling from integral membrane proteins to intracellular signaling partners. Thus, many PDZ domain proteins also play decisive roles in diseases and hence such proteins are relevant targets in drug discovery efforts. However, PDZ domains are also rather promiscuous proteins, often binding to several different partners, typically through their C-terminal region. There could therefore be concerns about achieving selectivity toward a specific PDZ domain. Moreover, it has also been realized that developing drug-like small-molecule inhibitors of PDZ domains is far from straightforward. A key issue is most likely the elongated binding pocket, where a C-terminal peptide is coordinated not only via the terminal carboxylate group, but also through a network of backbone hydrogen bonds, which seems to be difficult to mimic with small molecules. In accordance with this, the most advanced compounds targeting PDZ domain proteins are peptides. While peptides were previously not considered attractive drug candidates, we now see increasing numbers of peptides in clinical development in general, and peptides even have higher success rates than small molecules. As outlined in our review, there are several promising PDZ domain proteins, which can be targeted with therapeutic peptides or potentially small molecules.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

drug targets, inhibitor, PDZ, protein–protein interactions

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