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Review

Tetraspanins: structure, dynamics, and principles of partner-protein recognition

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Tetraspanins are a large, highly conserved family of four-pass transmembrane (TM) proteins that play critical roles in a variety of essential cellular functions, including cell migration, protein trafficking, maintenance of membrane integrity, and regulation of signal transduction. Tetraspanins carry out these biological functions primarily by interacting with partner proteins. Here, we summarize significant advances that have revealed fundamental principles underpinning structure–function relationships in tetraspanins. We first review the structural features of tetraspanin ectodomains and full-length apoproteins, and then discuss how recent structural studies of tetraspanin complexes have revealed plasticity in partner-protein recognition that enables tetraspanins to bind to remarkably different protein families, viral proteins, and antibody fragments. Finally, we discuss major questions and challenges that remain in studying tetraspanin complexes.

Highlights

Tetraspanins regulate signal transduction by interacting with partner proteins belonging to remarkably different protein families, including extracellular enzymes, integrins, members of the immunoglobulin superfamily, and intracellular signaling proteins.

Structures of full-length tetraspanins have revealed a common overall architecture, with a cone-shaped transmembrane (TM) domain containing an intramembrane binding pocket. This pocket can bind lipids, which appear to modulate tetraspanin function.

Many tetraspanins are conformationally dynamic, existing in at least two states with distinct TM conformations and ectodomain orientations.

The molecular association of a tetraspanin with its partner can be mediated through the large extracellular loop (EC2 domain) and/or the TM domain. The dependency for each region differs based on the bound partner.

Tetraspanins: a conserved family of four-pass TM proteins with critical cellular functions

Tetraspanins are an exceptionally well conserved family of four-pass TM proteins – numbering 33 unique members in humans – that have essential functions in many different cellular contexts. Tetraspanins first arose in unicellular eukaryotes, where they were thought to regulate plasma membrane dynamics and structure [1]. During evolution, this function was likely repurposed to mediate cell–cell interactions, linking the emergence of tetraspanins to the transition from unicellularity to multicellularity [2]. In multicellular organisms, the requirement for specialized cell types likely selected for the duplication and differentiation of tetraspanins that evolved to have unique cellular functions [2]. In humans, the first tetraspanins were discovered on the surface of human leukocytes [3]. Tetraspanins were later identified in nearly every cell type [4], each of which usually expresses multiple family members. Tetraspanin expression varies considerably among family members and by cell type, ranging between approximately 3000 and 100 000 copies per cell [4,5]. While some tetraspanins are widely expressed (e.g., CD81 on nearly all cell types), others have restricted expression on a single cell type (e.g., peripherin/RDS on rod outer segments in the retina [6] and CD37 on leukocytes [7]).

Although tetraspanins share a common ancestry, they acquired various discrete biological functions. Knockout and mutagenesis studies in mice and other organisms have identified broad and critical requirements for tetraspanins in the immune, hematopoietic, reproductive, genitourinary, epithelial, visual, and auditory systems. For example, mutations in peripherin/rds lead to retinal dystrophies, and a homozygous null mutation in mice shows disrupted photoreceptor morphogenesis [8,9]. Alternative splicing of CD81 leads to profound defects in B cell development and common variable immunodeficiency [10,11]. Frameshift mutations in CD151 lead to a bleeding disorder accompanied by hereditary nephritis and skin blistering, consistent with the role of CD151 in supporting the function of integrins, which bind to laminin, an essential component of the basement membrane [12,13]. Knockouts of Tspan7 and Tspan4 in zebrafish lead to an

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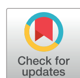

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inability to form migrasomes, a newly discovered organelle in migrating cells, and impaired organ morphogenesis [14]. Additionally, CD9 null mice are sterile due to failure of sperm–egg fusion [15,16], and CD63 knockout mice show increased urinary flow, water intake, and reduced urine osmolality [17]. In contrast to these mutations leading to loss-of-function phenotypes, other knockout studies have relatively mild phenotypes, suggesting that some tetraspanins can functionally compensate for the knockout of a similar member. In this review we highlight advances in our understanding of the fundamental principles underlying the structure and function of tetraspanins. We begin by examining the structural characteristics of tetraspanin ectodomains and full-length apoproteins, and then discuss recently solved structures of tetraspanin complexes, which have uncovered the ability of tetraspanins to recognize structurally diverse classes of partner proteins, viral proteins, and antibody fragments.

Glossary

Cysteine-cysteine-glycine (CCG) motif: a conserved motif found in the large extracellular loop of all tetraspanins.
LEL (EC2): large extracellular loop, or second extracellular domain of tetraspanins.
Lipidic cubic phase (LCP) crystallography: a method for membrane protein structure determination using a membrane-mimetic matrix.
SEL (EC1): small extracellular loop or first extracellular domain of tetraspanins.
Tetraspanin-enriched microdomains (TEMs): specialized membrane domains enriched in tetraspanins and their transmembrane partner proteins.

Tetraspanins interact with partner proteins to modulate signal transduction

Whereas many multipass TM proteins, such as G-protein-coupled receptors (GPCRs) or transporters, bind small molecules or protein ligands to execute their functions, tetraspanins do not have an obvious receptor function. Instead, tetraspanins bind specific partner proteins to facilitate their localization in the membrane and association with signaling effectors. In many cases, tetraspanins are also required for the cotransport of their interaction partner through the secretory pathway, enabling the expression of the mature partner protein on the cell surface [1,10]. Partner proteins depend on tetraspanins for their proper function, highlighted by the fact that tetraspanin knockout phenotypes often mimic the partner-protein knockout phenotype [2]. In addition to regulating the membrane organization of their partner proteins, tetraspanins may influence signal transduction by facilitating signaling activity of their partner proteins (e.g., CD81-CD19), or by direct interaction of their cytoplasmic tails with kinases and phosphatases [18–20]. It is also possible that some tetraspanins connect to signaling effectors by binding to adaptor proteins. The N terminus of CD53, for example, interacts with protein kinase C (PKC) β upon B cell receptor stimulation [21], even though its N-terminal sequence does not have any known PKC-binding motifs [21]. Instead, it is possible that the C terminus PDZ-binding motif of CD53 interacts with cytosolic proteins containing a PDZ domain that could then indirectly recruit PKC [21]. Another possibility is that tetraspanins maintain a local lipid and protein composition that favors PKC binding instead of direct recruitment of signaling molecules via their cytoplasmic tails [21].

Shortly after tetraspanins were first discovered [3], they were proposed to act as 'molecular facilitators' [4], compartmentalizing specific partner proteins in the membrane to form stable and functional signaling complexes. At the same time, the concept of a 'tetraspanin network' was also introduced, drawing on evidence that tetraspanins coupled to both specific, non-tetraspanin partner proteins and also to other tetraspanins, allowing them to organize membrane microdomains of a particular composition [5]. These microdomains have been termed **tetraspanin-enriched microdomains (TEMs)** (see Glossary) or tetraspanin-enriched nanodomains; they compartmentalize specific lipids, tetraspanins, and their interacting partner proteins into domains 100–200 nm in size [6,7,22]. These microdomains are essential for the spatial organization of signaling molecules in the membrane and, more recently, were shown to be critical for membrane repair by forming rigid ring-like structures that act as physical barriers to maintain membrane integrity after cellular damage [23,24].

Tetraspanins interact with partners belonging to at least four remarkably different groups: extracellular enzymes (e.g., ADAM metalloproteases), integrins and other adhesion proteins, members of the immunoglobulin superfamily (e.g., CD19 and CD4), and intracellular signaling proteins (e.g., PKC) [2]. The best-studied tetraspanin-partner interaction is that of CD81 with the B cell coreceptor CD19. CD81 acts as a molecular chaperone to traffic mature, properly folded

CD19 to the plasma membrane [10]. In addition, CD81 also regulates the diffusion of CD19 by immobilizing CD19 and associated signaling scaffolds in distinct locations in the membrane, thereby regulating the signaling of CD19 during B cell activation [25]. These functions of CD81 highlight two critical functions of tetraspanins: both as regulators of intracellular trafficking, and as scaffolding proteins on the plasma membrane that organize partners into functional signaling complexes. While some interactions with partners are specific to a single tetraspanin (e.g., CD19-CD81), tetraspanins also share common partners (e.g., EWI-2 with both CD9 and CD81, and ADAM10 with six members of the tspanC8 family) [26,27].

Characteristic structural features of tetraspanins

Although several classes of membrane proteins contain four TM domains, including claudins [28], connexins [29], and the CD20-like family [30], tetraspanins are distinguished by a unique cysteine pattern within their ectodomain which contains between two and four stabilizing disulfide bonds and a conserved **cysteine-cysteine-glycine (CCG) motif**. These disulfides are essential for the correct folding of the ectodomain, and the number of disulfide bonds within the ectodomain is used to classify tetraspanins into three main groups, with two, three, or four disulfide bonds (Table 1).

Tetraspanins are small, 22–39 kDa in mass, and they contain four main functional domains: the TM region, two distinct extracellular loops, and short N- and C-terminal cytoplasmic regions (Figure 1A). The TM domain makes up nearly half of the molecular weight of a tetraspanin protein. It is the most highly conserved region of the molecule, consistent with an essential role in function (Figure 1B) [18,31]. In addition, tetraspanins have two unequally sized ectodomains, the large and the small extracellular loops. The small extracellular loop (**SEL** or EC1) ranges from 13 to 31 amino acids in size and is positioned between TM1 and TM2. The large extracellular loop (**LEL** or EC2), 69–132 amino acids in length, protrudes from the cell surface between TM3 and TM4. This region is the site of maximum variability among tetraspanins and appears to be the primary determinant of partner-protein specificity for many tetraspanin complexes [32]. Tetraspanins also contain short N-terminal and C-terminal cytoplasmic tails, which are also highly divergent among different tetraspanins, sharing 21–38% identity, respectively [18]. In some tetraspanins, these

Table 1. The three groups of tetraspanins are divided into three groups based on the number of disulfide bonds in the EC2 domain

Two disulfides (Group 1)	Three disulfides (Group 2)	Four disulfides (TspanC8)
• CD81 • CD9 • CD53 • Tspan2 • Tspan32	• UP-1a • UP-1b • Rom-1 • Peripherin-2 • CD63 • CD151 • CD82 • CD37 • Tspan1 • Tspan3 • Tspan4 • Tspan6 • Tspan7 • Tspan8 • Tspan9 • Tspan11 • Tspan12 • Tspan16 • Tspan18 • Tspan19	• Tspan5 • Tspan10 • Tspan13 • Tspan14 • Tspan15 • Tspan17 • Tspan31 • Tspan33

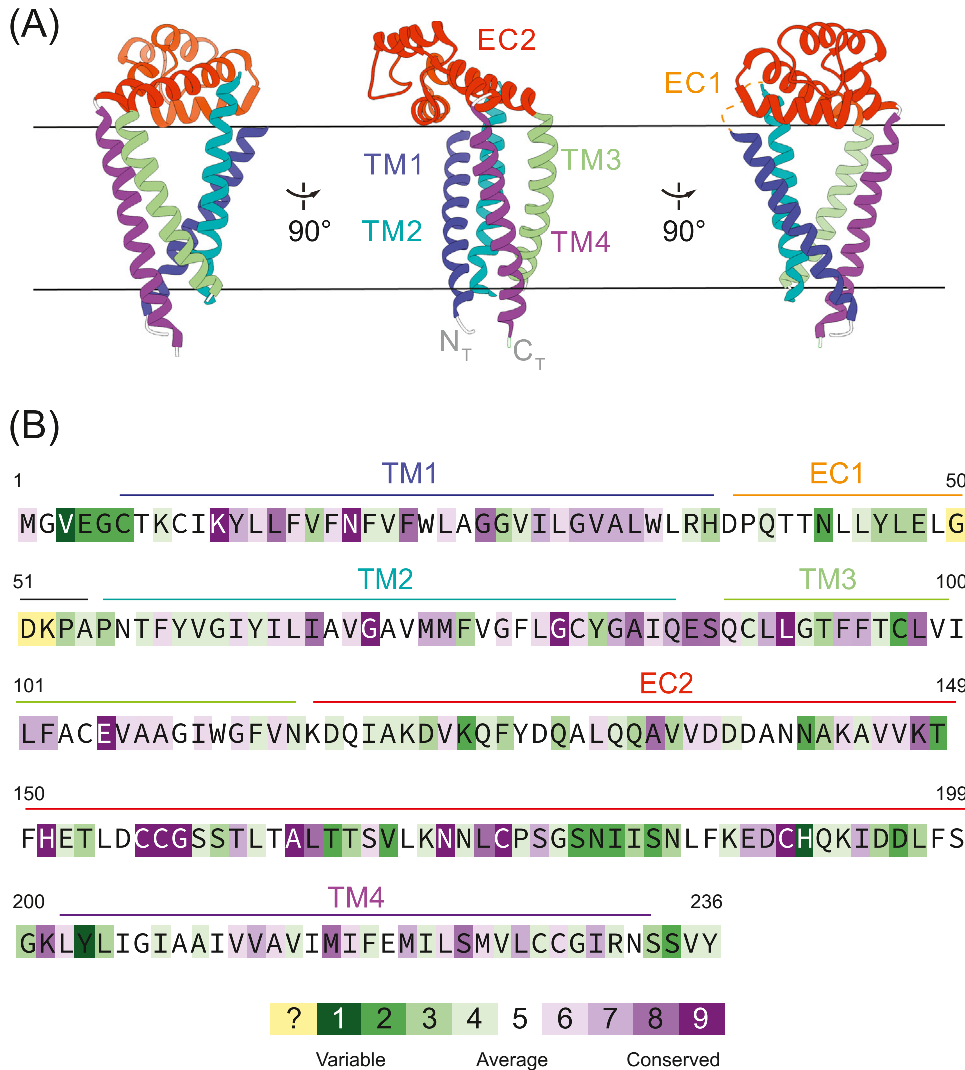

Figure 1. Overall tetraspanin domain organization and sequence conservation. (A) The structure of full-length CD81 [Protein Data Bank (PDB) 5TCX], highlighting the four transmembrane (TM) segments, large extracellular loop (EC2 domain), and the small extracellular loop (EC1 domain). (B) CD81 sequence colored by residue conservation among the 32 human tetraspanin paralogs. Residues are colored on a sliding scale from purple (highly conserved) to green (poorly conserved). Residues with insufficient information for analysis are colored yellow. Conservation was determined using the Consurf server [74].

intracellular tails contain targeting motifs to intracellular compartments or internalization signals for endocytic trafficking [1]. For example, the tetraspanin CD63 has a short GYEVM motif within its C-terminal tail that is essential for trafficking to lysosomes [33].

The first tetraspanin structure: the CD81 EC2 domain

Recent advances in tetraspanin structural biology have begun to reveal how tetraspanins function at the molecular level. Until recently, tetraspanin structural biology focused almost exclusively on the CD81 ectodomain because of its well characterized biology and its amenability to crystallography. Within the past 5 years, the focus of tetraspanin structural biology has shifted to studying full-

length tetraspanins and tetraspanins in complex with partner proteins. The recent determination of the first full-length tetraspanin structures [34,35] and structures of tetraspanins in complex with binding partners [35–37] represent milestones for the tetraspanin field. These structures have provided the first molecular views of tetraspanins in multiple distinct functional states, including two states classified as 'open' and 'closed' conformations based on the orientation of the TM helices and the orientation of the ectodomain relative to the TM domain. Table 2 lists all tetraspanins for which structures have been determined to date, with their Protein Data Bank (PDB) and/or electron microscopy data bank (EMDB) ID codes.

Table 2. PDB and/or EMDB codes for all reported tetraspanin structures to date

Structure	Method	PDB/EMDB code	Refs
CD81 large extracellular loop	X-ray crystallography	1G8Q	[38]
CD81 large extracellular loop (new crystal form)	X-ray crystallography	1IV5	[42]
Tetraspanin uroplakins in complex with UP11 and UP111a	Single particle cryoEM	N/A	[67]
TSP2 from *Schistosoma mansoni*	NMR	2M7Z	[70]
Human, mouse, and African green monkey CD81 large extracellular loop	X-ray crystallography	3X0E, 3X0F, 3X0G	[71]
CD81 large extracellular loop in complex with K04 or K13 Fab	X-ray crystallography	5DFV, 5DFW	[45]
Full-length CD81	X-ray crystallography (lipidic cubic phase)	5TCX	[34]
CD81 extracellular loop in various conformations	X-ray crystallography	5M33, 5M2C, 5M3T, 5M3D, 5M4R	[46]
CD81 large extracellular loop in complex with scFV fragment 4, 5 or 10	X-ray crystallography	6EJG, 6EJM, 6EK2	[43]
CD81 large extracellular loop in complex with 5A6 Fab	X-ray crystallography	6U9S	[44]
Full-length CD9	X-ray crystallography (lipidic cubic phase)	6K4J	[35]
CD9-EWI-2 complex	Single particle cryoelectron microscopy	EMD-30026, EMD-30027	[35]
CD9 large extracellular loop	X-ray crystallography	6RLR	[36]
CD9 large extracellular loop in complex with nanobody 4E8 or 4C8	X-ray crystallography	6Z1V, 6Z20	[36]
CD9-EWI-F complex	Single-particle cryoelectron microscopy	EMD-11053	[36]
Full-length CD53	X-ray crystallography (lipidic cubic phase)	6WVG	[39]
CD81-CD19 complex	Single-particle cryoelectron microscopy	7JIC, EMD-22344	[37]
CD81 large extracellular loop in complex with HCV envelope glycoprotein E2	X-ray crystallography	7MWW, 7MWS, 7MWX	[72]
Tspan15 large extracellular loop	X-ray crystallography	7RDB, 7RD5	[73]
Rod outer segment disk rims with peripherin-2 and ROM1 assemblies	Cryoelectron tomography	EMD-13321, EMD-13322, EMD-13323, EMD-13324	[54]
Tspan15-ADAM10 complex	Single-particle cryoelectron microscopy	8ESV, EMD-28580	[69]
ROM1-peripherin-2 complex	Single-particle cryoelectron microscopy	7ZW1, EMD-14991	[66]

The first structure of any tetraspanin domain, reported in 2001, was an X-ray crystal structure of the large extracellular loop (EC2) of human CD81 [38]. This structure revealed a fold with five α-helices (helices A–E) (Figure 2A). Helices A, B, and E make up a constant region, also referred

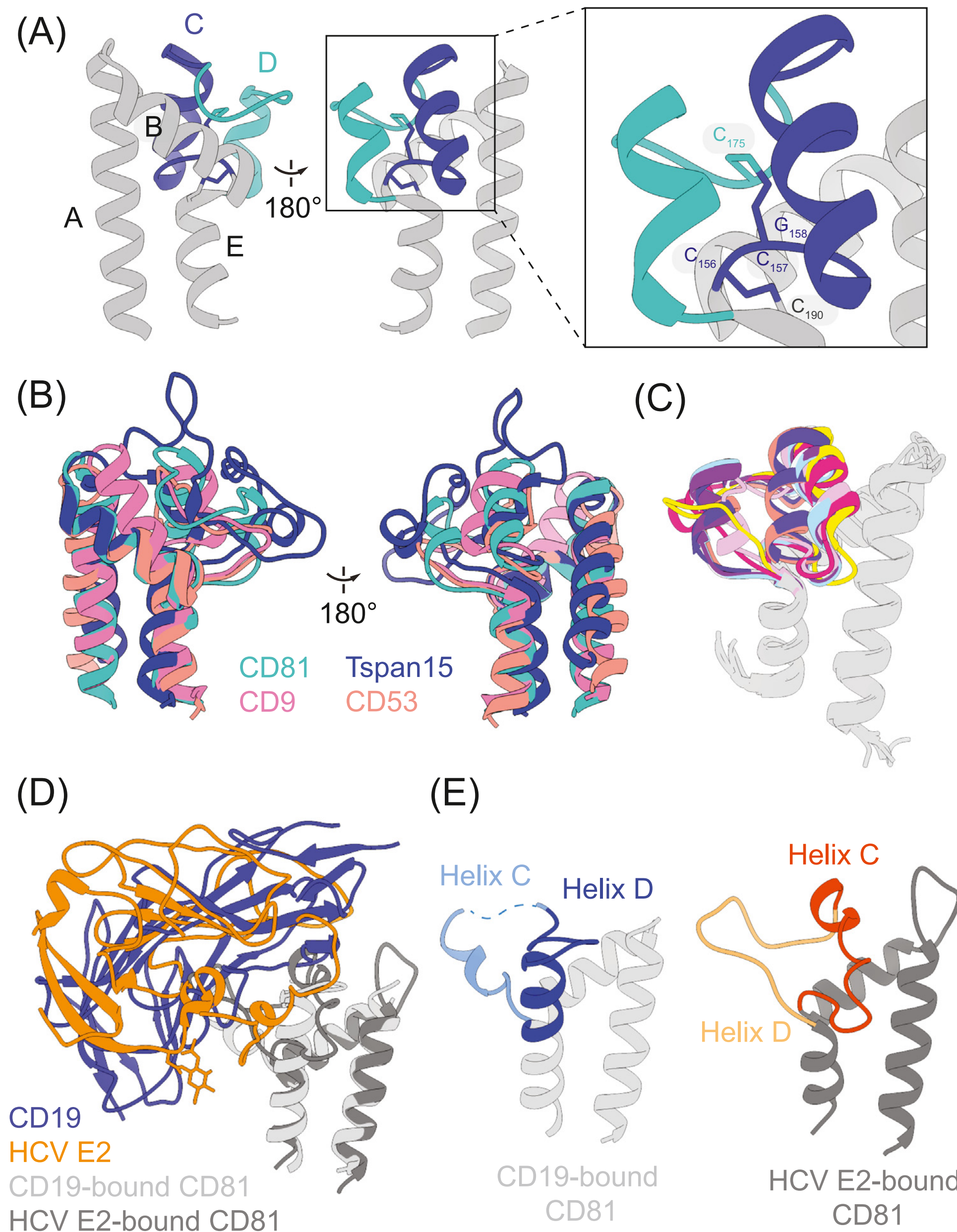

Figure 2. Architecture and dynamics of the EC2 domain. (A) Architecture of the EC2 domain of CD81, highlighting the C–D helices (colored blue and teal) on top of a conserved stalk region made up of helices A, B, and E (colored gray). The disulfide bond formed from residues in the conserved cysteine-cysteine-glycine (CCG) motif are shown in the zoom panel. (B) Overlay of CD81, CD9, CD53, and Tspan15 EC2 crystal structures [Protein Data Bank (PDB) codes 5TCX, 6K4J, 6WVG, and 7RDB]. (C) Overlay of several CD81 EC2 structures, highlighting the plasticity of the C–D helices (colored) and the rigid stalk region (gray). (D) Superimposition of the CD19-CD81 complex (PDB 7JIC) and the hepatitis C virus (HCV) E2-CD81 complex (PDB 7MWX). (E) Comparison of the orientation of the C–D helices in the CD19-bound and E2-bound structures.

to as the 'stalk', which is rigid and relatively conserved in sequence among human tetraspanin paralogs. A smaller 'head' region, consisting of helices C and D, sits directly on top of the stalk region and is substantially more variable in sequence and in size. This head region is stabilized by two disulfide bonds originating from two adjacent cysteine residues (C156 and C157) of the conserved CCG motif (Figure 2B), and is composed of variable stretches of amino acids separated by a variable number of cysteines, each yielding a predicted secondary structure unique for each tetraspanin. Sequence analysis suggested that this five-helix fold would be conserved within the family [38], and in line with this prediction, all other ectodomain structures of human tetraspanins reported to date show a remarkably similar fold within the rigid stalk region, with major structural variations appearing only in the C-D helix region (Figure 2C) [35,36,39]. This hypervariable region represents the key site in which structural variability in tetraspanins encodes specificity for their interactions with partners. For example, the C-D helices of CD81 are necessary for CD19 trafficking to the cell surface, and the association of the tetraspanin CD151 with α3 integrin is dependent on residues 185–196 within helices C and D [40,41].

Helices C and D within the EC2 domain are highly dynamic

Subsequent crystal structures of the CD81 EC2 domain in a different space group [42], bound to antibody fragments [43–45], along with molecular dynamics simulations at different pH [46] revealed that the C-D helix 'head' region is highly dynamic (Figure 2C). This dynamism underlies a unique aspect of tetraspanin function: that a single tetraspanin can form direct partnerships with structurally distinct partners. For example, CD81 associates with CD4 and CD8 in T cells [47,48] but associates with CD19 in B cells [49] and the TM proteins EWI-2 and EWI-F on nearly all cell types [27]. CD81 is also the cellular receptor for a completely different type of partner: the hepatitis C virus (HCV) envelope protein E2 [50,51]. To date, the greatest insight into the structural origin of the occurrence of several partners common to a single tetraspanin comes from the structures of the CD19-CD81 complex and the CD81-HCV E2 complex (Figure 2D). These structures reveal extreme plasticity within the C-D helices of the EC2 domain, allowing CD81 to dynamically reorient to recognize two structurally distinct, yet common partners. When bound to CD19, helix C of CD81 unwinds to create a CD19-binding surface, and helix D merges with helix E to create a longer helix that becomes continuous with the fourth TM domain (Figure 2E). By contrast, helix C and D adopt structurally distinct conformations when bound to E2, with the C helix remaining mostly structurally unchanged and the D helix adopting an extended conformation to enable E2 binding (Figure 2E).

Tetraspanins share a conserved cone-shaped architecture of their TM domain

More than a decade after the first structure of the CD81 ectodomain was reported, the crystal structure of full-length CD81 was determined, the first full-length structure of any member of the tetraspanin family [34]. This key advance, which elucidated the overall domain organization of the family, was enabled by **lipidic cubic phase (LCP) crystallography**, which allows for crystallization in a lipid bilayer that mimics the native membrane environment [52]. Several years later, the structures of CD9 [35] and CD53 [39] were also determined using LCP crystallography, bringing the total number of full-length tetraspanin apoprotein structures to three out of the 33 tetraspanins found in humans.

These three full-length structures revealed a general architecture of the tetraspanin family, in which the TM domain resembles a cone where the intracellular ends are tightly bundled (Figure 3A). The TM domain is directly capped by the EC2 domain, which protrudes only several nanometers above the plasma membrane. The four TM helices are arranged in two separated pairs of asymmetric, antiparallel helices (TM1/TM2 and TM3/TM4), which converge only near the cytoplasmic side of the membrane to create a large (~3000 A^2) central cavity. This fold

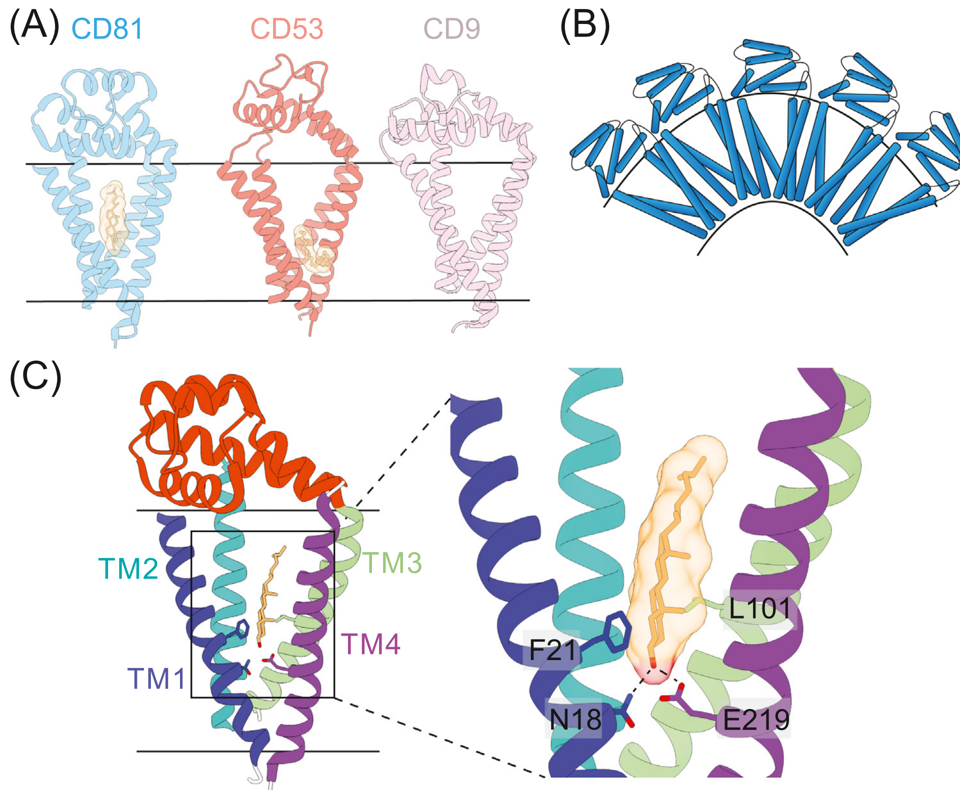

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Figure 3. Architecture of the transmembrane (TM) domain. (A) X-ray crystal structures of full-length CD81, CD53, and CD9, showing the cone-shaped architecture of the transmembrane domain. Modeled lipids are shown in yellow. Density for a lipid was observed in the CD9 the F_o–F_c map, but was not modeled in the structure. (B) Schematic illustrating how the cone-shaped transmembrane domain can generate positive membrane curvature. (C) CD81-cholesterol interactions. Several residues interacting with cholesterol are rendered as sticks and labeled in the zoomed-in view.

does not resemble that of any other integral membrane protein of known structure, and is dramatically different from the previously predicted tightly packed orientation of the TM region [53]. CD9 crystallized in a wave-like lattice, and some have suggested that this cone-shaped architecture could play a role in generating curvature in the membrane, explaining how the clustering of tetraspanins generates regions of local curvature within the membrane (Figure 3B) [35]. Support for this came from cryoelectron tomography studies of mouse rod outer segments, which showed that a continuous assembly of V-shaped complexes of the tetraspanins peripherin-2 (PRPH2) and rod outer segment membrane protein-1 (ROM1) enforces an extreme radius of curvature at the disk rims [54]. Additionally, using a biomimetic system of membrane tubules with controlled curvatures, the TM domain of tetraspanins was recently shown to be a curvature sensor with a preference for positive membrane curvature [55].

Tetraspanins contain a hydrophobic intramembrane binding pocket

The most striking feature of the full-length CD81 structure was a bound cholesterol molecule within the intramembrane cavity, and the later structures of CD9 and CD53 also showed density consistent with that of a bound lipid in the same cavity (Figure 3A) [34,35,39]. While the bound lipid seen in the CD9 and CD53 structures was not definitively identified, radioligand binding assays were used to confirm that CD81 specifically binds to cholesterol but not to other lipids

of similar sizes, such as palmitate or estradiol [34]. Cholesterol binds within a large central cavity of CD81, surrounded by hydrophobic residues from each of the four TM helices (Figure 3C). There are also two polar residues, asparagine 18 (N18) and glutamate 219 (E219), belonging to TM1 and TM4, respectively, that form hydrogen bonds to the hydroxyl group of cholesterol. These cholesterol-interacting residues are highly conserved throughout evolution, with 27 of the 33 human tetraspanins containing an asparagine at position 18 [34]. Although E219 is found in only one other human tetraspanin (Tspan10), the majority of human tetraspanins have a glutamate or glutamine residue nearby on the preceding turn of the helix, suggesting that lipid binding within the intramembrane cavity may be a general feature of the family [34].

Identifying a lipid-binding pocket within CD81 raised an important question: is the bound cholesterol a functionally relevant, modulatory lipid, or a structural lipid observed only due to the presence of cholesterol in the crystallization mix? Several lines of evidence support the idea that lipids can play a modulatory role in tetraspanin function. In the early 2000s, the first suggestion of a link between cholesterol and tetraspanins came from the observation that tetraspanin–tetraspanin complexes could not be observed after lysis with digitonin, a cholesterol-sequestering agent [56]. Photoactivatable cholesterol was then used to show that the tetraspanins CD9, CD81, and CD82 are in close proximity to cholesterol in the membrane [56]. Cholesterol was also necessary for maintaining a CD81 monoclonal antibody epitope and CD81-dependent infection by *Plasmodium falciparum* sporozoites [57]. Additionally, a mutant of CD81 that favors the cholesterol-bound state showed enhanced HCV binding, consistent with cholesterol sensing playing a key role in tetraspanin function [58]. While these studies hinted at a functional interplay between tetraspanin function and cholesterol, mutagenesis of the cholesterol-binding residues identified from the CD81 crystal structure was the first demonstration that cholesterol binding and partner protein engagement are functionally linked. A CD81 construct with an alanine mutation of a cholesterol-binding residue, E219, trafficked more CD19 to the cell surface than wild-type CD81, indicating that cholesterol-free CD81 exhibits a higher affinity for its molecular partner, CD19 [34]. However, another study in a different cell line did not observe a substantial difference in CD19 surface delivery [58]. It is not clear whether the different findings reflect differences in methodology, differences in cellular context, or differences in the abilities of the two systems used to elicit differences above background activity in CD19 surface export. Molecular dynamics also has suggested that cholesterol-free CD81 adopts an 'open' conformation in which EC2 separates from the TM domain, suggesting for the first time that tetraspanins might exist in multiple different functional conformations which are directly influenced by lipid binding [34]. While much attention has been paid to the role of cholesterol binding in CD81 function, it remains to be determined whether the function of other family members is modulated by lipid binding.

Tetraspanins contain membrane-proximal palmitoylated cysteines

In addition to noncovalent interactions with lipids, palmitoylated cysteines are also considered important for the interaction of tetraspanins with partner proteins and the formation of tetraspanin microdomains. Palmitoylation is a reversible post-translational modification that consists of the attachment of a saturated fatty acid chain (palmitate) to cysteines situated up to 8 Å into the inner leaflet of the membrane [59,60]. A unique feature of the CD9 structure is the extra densities near intracellular ends of the TM helices, which are likely derived from attached palmitoyl groups [35]. The analogous cysteines were mutated in the CD81 construct to produce a more homogenous sample for crystallization [34], but the CD9 structure suggests that these modifications could play a role in anchoring the TM helices in the membrane, suggesting that palmitoylation could have a role in stabilization of the cone-shaped architecture of the TM helices.

In one study, palmitoylation states of the tetraspanin CD9 were profiled using high-resolution native mass spectrometry [61]. CD9 was found to have nonstochastic distributions of palmitoylated cysteines, and the three most frequently palmitoylated sites were found to be required for binding to its molecular partner, EWI-F [61]. However, no specific cysteines were required for binding to a different molecular partner (EWI-2), indicating apparent differences in lipidation states based on the partner protein bound. Other studies have shown that loss of palmitoylation also results in decreased lateral associations of the tetraspanin CD151 with other CD151 molecules [62], of CD9 with the tetraspanins CD81 and CD53 [63], and β4 integrins with the CD81, CD9, and CD63 [64]. Although palmitoylation has been confirmed for well characterized tetraspanins [63], further work is needed to characterize its precise effects on tetraspanin surface distribution, conformational states, and partner binding.

Plasticity in tetraspanin-partner interactions

Elucidation of the structure of tetraspanins with various partner proteins will be essential for understanding their function as signaling facilitators and the molecular basis for partner protein specificity. Recently, structures for several tetraspanin-partner complexes were reported, revealing that tetraspanins are remarkably plastic in their recognition of a partner, and there is not one defining principle of how partners are recognized [35–37,65,66].

The structure of the CD19-CD81 B cell coreceptor complex was the first complex reported at a resolution sufficient to build a complete atomic model. This structure captured the first snapshot of a tetraspanin in an 'open' conformation, in which the TM helices rearrange to occlude a central lipid-binding cavity, and the ectodomain swings 60° away from the membrane to position helices A and E of the ectodomain to be near-continuous with TM3 and TM4, respectively (Figure 4A,B). The C–D helices rearrange dramatically within the ectodomain to create the CD19 binding pocket. This conformation is stabilized by rotation across the disulfide bonds of the CCG motif, establishing a structural role for this conserved motif. This structural data suggests a conformational switch model, in which the tetraspanin ectodomain is reoriented away from the membrane upon partner recognition to stabilize the partner-protein ectodomain, and the TM domain reorients to occlude a lipid-binding cavity, potentially resulting in relocalization of the partner protein into a different membrane microdomain. In line with this conformational switch model, the CD19-CD81 interaction was shown to be highly dynamic, with CD81 disengaging from CD19 after B-cell activation [44]. Important areas of future investigation will be to determine whether other tetraspanin-partner complexes are constitutive or dynamic in nature, and to elucidate structural features that underly any dynamism (see Outstanding questions).

Similarly, the cryoelectron microscopy (cryoEM) structure of the tetraspanin uroplakins (UP1a and UP1b) with their single-pass TM protein partners UPII and UPIIIa displays a remarkably similar topology to the CD19-CD81 complex, with the tetraspanins arranged in an 'open' conformation, tightly bundled TM helices, and an extended ectodomain [67]. Likewise, the cryoEM structure of a heterodimer of two retina-specific tetraspanin family members, peripherin (PRPH2) and ROM1, was recently reported [66]. Like the CD19-CD81 and uroplakin complexes, the TM domains of PRPH2 and ROM1 are tightly packed and do not contain central cavities (Figure 4C,D). PRPH2 and ROM1 dimerize through direct contact between the C–D helices of their EC2 domains, consistent with this region being a key determinant for partner specificity.

In contrast to the structures of the CD19-CD81, PRPH2-ROM1, and uroplakin complexes, the structures of CD9 with EWI2 and EWI-F and of Tspan15 with the metalloprotease ADAM10 display remarkably different architectures and modes of partner protein recognition. Two independent cryoEM structures of the CD9-EWI2 and CD9-EWI-F complexes were recently reported

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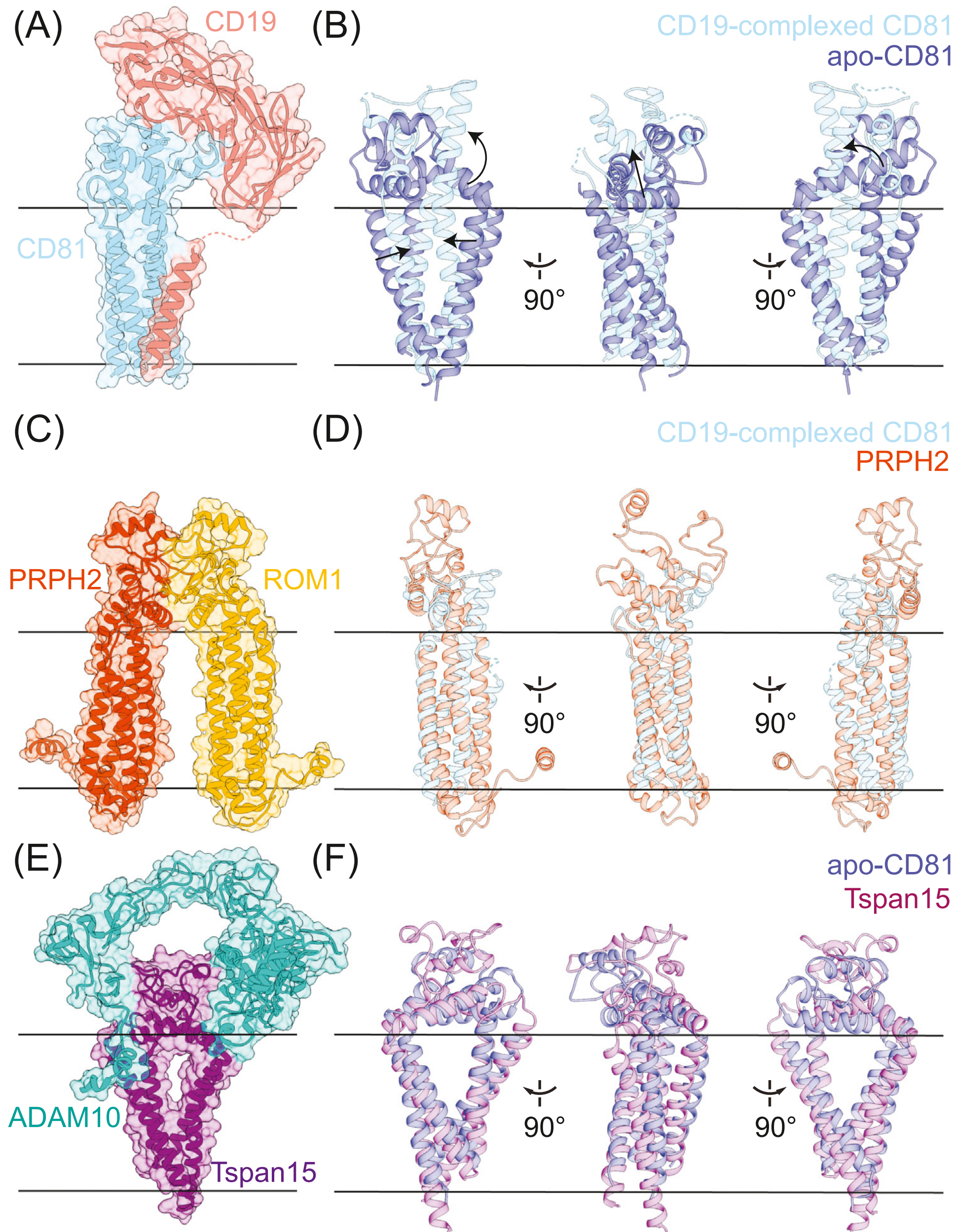

Figure 4. Structures of tetraspanins with their partner proteins. (A) Cryoelectron microscopy (cryoEM) structure of the CD19-CD81 structure [Protein Data Bank (PDB) 7JIC]. (B) Alignment of apo-CD81 ('closed' state, PDB 5TCX) and CD19-complexed CD81 ('open' state). (C) CryoEM structure of the peripherin 2-ROM1 heterodimer (PDB 7ZW1). (D) Alignment of peripherin 2 (PRPH2) and CD19-complexed CD81. (E) CryoEM structure of ADAM10-Tetraspanin 15 complex (PDB 8ESV). (F) Alignment of apo-CD81 with tetraspanin 15.

[35,36]. While the moderate resolution of these structures prevented the building of an atomic model, they displayed an overall topology remarkably different from the 'open' conformation of the tetraspanins seen in the previously discussed complexes. The CD9-EWI-2/F complex is arranged in a heterotetrameric architecture, with two CD9 protomers sandwiching an EWI-2/F

dimer in the center. Unlike other tetraspanin-partner pairs, the primary contact interface appears to be within the TM domains instead of the EC2 domain. The arrangement of the TM helices of CD9 closely matches the cone-shaped architecture of the apo-CD9 crystal structure, suggesting that CD9 remains in the 'closed' state upon binding to its molecular partner EWI-2/F. The authors proposed that the cone-shaped architecture of the CD9-EWI-F complex and the exposure of a putative dimerization interface within the first two TM helices of CD9 results in the assembly of higher-order complexes, or TEMs [31,36]. This clustering of cone-shaped complexes could facilitate the physical bending of the membrane when the complex is in a higher oligomeric state, which is consistent with other reports implicating tetraspanins in the maintenance of membrane curvature [36]. In other tetraspanins, homodimerization sites have been mapped to helix D within the EC2 domain, suggesting that each tetraspanin has a unique mode of assembling into higher-order complexes [68].

Like the CD9-EWI2/F complexes, the Tspan15-ADAM10 complex displays a remarkably different overall architecture from the CD19-CD81 complex (Figure 4E,F) [69]. Tspan15 is in a 'closed' conformation, with the TM domain arranged in a cone and the EC2 domain sitting on top of the membrane. The primary ADAM10-Tspan15 interaction site is within the ectodomain, with ADAM10 interacting with two distinct regions of the C–D helices. This interaction stabilizes ADAM10 in an open, active conformation and positions the active site of ADAM10 about 20 Å from the membrane, explaining how a tetraspanin can tune the substrate specificity of the bound metalloprotease by altering the position of the ADAM10 active site relative to the membrane.

Comparison of the CD81 apoprotein structure with that of the CD81-CD19 complex also revealed that complex formation was associated with ordering of the EC1 loop [37], which becomes helical and interacts with EC2. EC1 is also visible in the structure of the CD53 apoprotein, which adopts an intermediate conformation between open and closed [39]. By contrast, the structures of Tspan15-ADAM10 [69] and CD9-EWI [35,36], complexes which have their tetraspanins in the closed state, do not show an interaction between EC1 and EC2. Together, these observations suggest that EC1 interactions with EC2 might promote a preference for the open tetraspanin conformation.

Concluding remarks

Work in the past several years has yielded important insights into the structural features underlying tetraspanin function. Structures of full-length tetraspanins bound to several different partner proteins have revealed that some tetraspanins appear to function as conformational facilitators to stabilize their partners in a specific conformation (e.g., Tspan15-ADAM10), while other tetraspanins seem to facilitate membrane localization of their partners during signal transduction through conformational switching between the closed and open states, while leaving the conformation of the partner unchanged (e.g., CD81-CD19). Future work determining the structures of tetraspanins with other molecular partners – such as integrins, other members of the immunoglobulin superfamily, and intracellular signaling molecules – will be needed to fully delineate this remarkable protein family's structural plasticity (see Outstanding questions).

Outstanding questions

Why are tetraspanins 'closed' in some structures and 'open' in others? Are tetraspanins intrinsically dynamic proteins, or are conformational dynamics a feature of only a subset of the family, with other family members adopting fixed states?

Do the 'open' and 'closed' conformations of tetraspanins support specific partner–protein interactions, are they an 'on/off switch' for partner binding, or do the conformational dynamics of tetraspanins have different roles in different tetraspanins?

Which tetraspanins, if any, form constitutive complexes with their partners?

What is the role of lipid binding in tetraspanin function and dynamics? Are all members of the family capable of binding lipids?

What is the structural basis for engagement of several different partner proteins by a single tetraspanin?

Does the ability of the TM domain of tetraspanins to sense and generate curvature direct the localization of partner proteins to specific membrane microdomains?

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Declaration of interests

A.C.K. is a cofounder and consultant for Tectonic Therapeutic and Seismic Therapeutic and for the Institute for Protein Innovation, a nonprofit research institute. S.C.B. is on the board of directors of the nonprofit Institute for Protein Innovation and the Revson

Foundation, is on the scientific advisory board for and receives funding from Erasca, Inc., for work unrelated to this review, is an advisor to MPM Capital, and is a consultant for IFM, Scorpion Therapeutics, Odyssey Therapeutics, Droia Ventures, and Ayala Pharmaceuticals for unrelated projects. K.J.S. has no interests to declare.

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