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Review Article

Molecular interactions shaping the tetraspanin web

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To facilitate the myriad of different (signaling) processes that take place at the plasma membrane, cells depend on a high degree of membrane protein organization. Important mediators of this organization are tetraspanin proteins. Tetraspanins interact laterally among themselves and with partner proteins to control the spatial organization of membrane proteins in large networks called the tetraspanin web. The molecular interactions underlying the formation of the tetraspanin web were hitherto mainly described based on their resistance to different detergents, a classification which does not necessarily correlate with functionality in the living cell. To look at these interactions from a more physiological point of view, this review discusses tetraspanin interactions based on their function in the tetraspanin web: (1) intramolecular interactions supporting tetraspanin structure, (2) tetraspanin–tetraspanin interactions supporting web formation, (3) tetraspanin–partner interactions adding functional partners to the web and (4) cytosolic tetraspanin interactions regulating intracellular signaling. The recent publication of the first full-length tetraspanin crystal structure sheds new light on both the intra- and intermolecular tetraspanin interactions that shape the tetraspanin web. Furthermore, recent molecular dynamic modeling studies indicate that the binding strength between tetraspanins and between tetraspanins and their partners is the complex sum of both promiscuous and specific interactions. A deeper insight into this complex mixture of interactions is essential to our fundamental understanding of the tetraspanin web and its dynamics which constitute a basic building block of the cell surface.

Introduction: spinning the tetraspanin web

Tetraspanins are highly conserved integral membrane proteins involved in membrane organization and compartmentalization. This is achieved through lateral interactions among tetraspanins and between tetraspanins and their partner proteins, thereby forming tetraspanin-enriched microdomains (TEMs), also referred to as 'the tetraspanin web'. The tetraspanin web creates a local environment containing a specific set of proteins in the plasma membrane which can facilitate or impede particular cellular processes [1].

Tetraspanins are small hydrophobic proteins that are ~200–350 amino acids in length and protrude 3–5 nm from the cell surface [2,3]. They consist of four transmembrane (TM) regions, one small extracellular loop (EC1) and one large extracellular loop (EC2), one small cytoplasmic loop and two short cytoplasmic tails. Although there are many different four-TM-domain proteins present on the cell surface, not all of these proteins are considered part of the tetraspanin superfamily. For example, the MS4A protein family comprises four-TM-domain proteins (including CD20 and HTm4), which are not considered to be genuine tetraspanins [4]. Genuine tetraspanins are characterized by the presence of a conserved CCG motif and two conserved cysteine residues located in the EC2 domain [5]. Tetraspanins have been shown to play a role in many vital cell biological processes, including adhesion, migration, fusion and signaling [6,7]. As a result of their involvement in these fundamental processes, tetraspanins have been implicated in the pathophysiology of numerous diseases, including HIV [8], hepatitis C [9], malaria [10], type 1 diabetes [11] and cancer [12]. The recent discovery that loss of a specific tetraspanin (CD37) leads to spontaneous development of B-cell lymphoma indicates

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that individual members of this family can have non-redundant functions in maintaining cellular health [13]. These studies demonstrate that cells rely on the organizational capacity of tetraspanins to regulate their function, which is inextricably tied to their ability to assemble the tetraspanin web.

The assembly of the tetraspanin web was proposed to be reliant upon three different levels of interactions, referred to as first-level (primary), second-level (secondary) and third-level (tertiary) interactions [7]. First-level interactions denote only tetraspanin–tetraspanin and tetraspanin–partner interactions that are direct in nature and resistant to disruption by strong detergents. Second-level interactions are only resistant to weak detergents and are formed when tetraspanins further interact by linking primary complexes to each other. Secondary interactions are at least partially dependent on palmitoylation since the removal of palmitoylation sites impairs the formation of second-level interactions between integrins and tetraspanins [14]. Third-level interactions are weak interactions which can only be detected in the presence of very mild detergents (e.g. CHAPS). Under these mild conditions, third-level tetraspanin complexes localize to low-density membrane fractions in a sucrose gradient [15,16]. This model for complex assembly based on a hierarchical approach allows for very dynamic and adaptable interactions between tetraspanins and other surface proteins. This model is, however, based on a rather descriptive division and says little about the functionality of these interactions. This review therefore classifies tetraspanin interactions based on their function in the formation of the tetraspanin web: interactions necessary to maintain tetraspanin structure, interactions that support tetraspanin web formation, interactions that add functional partners to the web and interactions that facilitate intracellular events.

Intramolecular interactions: supporting the structure of tetraspanins

The four TM domains that tetraspanins are named after are vital in determining the structure of these proteins. Several lines of evidence suggest that the four TM domains (20–25 amino acids in length) form a tightly packed bundle in the membrane [2,17]. However, this model is challenged by the first crystal structure of a full-length tetraspanin (CD81) as published by Zimmerman *et al.* [18]. A pair-wise packing of TM1 with TM2 and TM3 with TM4 was identified in a cone-shaped conformation wherein the pairs are in close proximity within the inner leaflet of the membrane and show further separation in the outer leaflet of the membrane (Figure 1). The results from evolutionary coupling analysis are consistent with this new model and suggest that TMs of other tetraspanins probably have a similar conformation [18]. Consistent with the idea that proper packing of the TMs is essential for the entire tetraspanin structure, mutations in TM domains were shown to affect the folding, stability and transport of several tetraspanins, e.g. uroplakin 1B [19], CD9 [17,20] and CD82 [21]. The molecular interactions enforcing proper packing are thought to be mediated by two highly conserved features in the TM domains of tetraspanins. The first is a set of polar residues (Asn, Gln and Glu) in TM1, TM3 and TM4 that are predicted to stabilize TM domain packing by polar interactions, hydrogen bonds and/or interactions with cholesterol [5,18,22]. The second is a conserved heptad repeat in TM1 and TM2 in which large hydrophobic residues in one TM domain are closely packed with small residues in the other TM domain (Figure 1) [17].

Next to the TM domains, folding of the EC2 is of particular importance since most tetraspanin intermolecular interactions are suggested to be mediated through this domain. Based on the crystal structure of the CD81 EC2 and the conservation of key features in other tetraspanins, the EC2 was proposed to contain five α-helices (A–E) forming a mushroom-shaped structure that can be divided in a rod-shaped 'stalk' region (A- and E-helices) and a 'head' region formed by the B-, C- and D-helices (Figure 1) [3]. Whereas the helical nature of the B- and C-helices was confirmed in a later NMR study, no secondary structure was found in the region of the D-helix [23]. Also, molecular dynamic simulations suggest that the region of the D-helix (and to a lesser extent the C-helix) is extremely flexible [24]. We will therefore refer to the region of the D-helix as the D-loop. Apart from the D-loop, the 'head' region has a rather defined structure. Structural integrity of the 'head' is mediated largely by the tetraspanin-wide conserved CCG motif that forms two disulfide bridges with two cysteine residues located at relatively fixed positions elsewhere in the EC2. These disulfide bridges were observed in both crystal structures and in an NMR structure of CD81 (EC2) [18,3,23], and have been reported to be important for proper CD81 and CD9 functioning [25,26]. The 'stalk' region is relatively rigid due to hydrophobic interactions and two salt bridges between the A- and E-helices [3]. Tight packing of the A- and E-helices was confirmed by NMR spectroscopy of the CD81 EC2 in solution [23], and conservation of involved residues suggests that this structural feature is shared between members of the tetraspanin family [27]. A recently proposed third factor influencing the structure (and thereby interactions with other proteins) of the EC2 is

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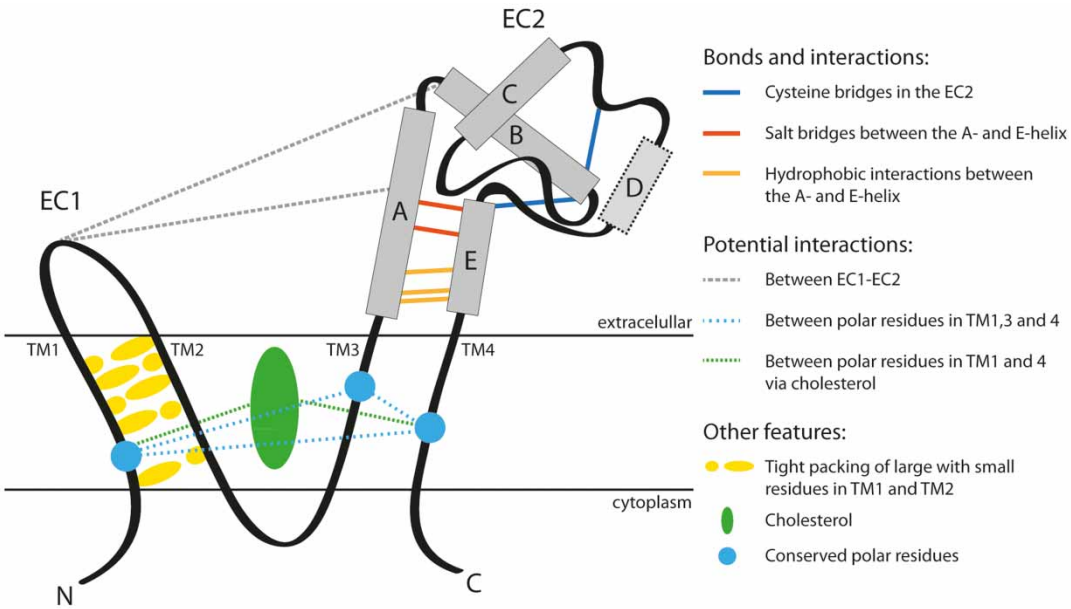

Figure 1. Intramolecular interactions of tetraspanins.

Model of the intramolecular tetraspanin interactions involved in the maintenance of its structure. The conformation of the large extracellular loop (EC2) is stabilized by salt bridges and hydrophobic interactions between the A- and E-helices in the 'stalk-region' and covalent cysteine bridges in the 'head-region' (B-, C-helices and D-loop). The conformation of the EC2 may also be stabilized by interactions with the small extracellular loop (EC1) under certain conditions. Inside the membrane, proper packing of the transmembrane domains (TM1–4) is assumed to be mediated by interactions between conserved polar residues in TM1, 3 and 4, which may be direct or indirect via the binding of cholesterol. Of note, the presence or absence of cholesterol is suggested to result in two different conformations of the tetraspanin, which are not visualized in this figure. Tight packing of TM1 with TM2 is facilitated by large and small residues opposing each other in a conserved heptad repeat motif.

the binding of cholesterol within the TM region of tetraspanins. Molecular dynamics simulations on the basis of the full-length CD81 crystal structure suggest that lack of cholesterol results in an extended or 'open' conformation of the EC2, whereas the EC2 stays close to the membrane in a 'closed' conformation when cholesterol is bound [18]. In the closed conformation, the EC2 is predicted to fold on top of the EC1 which is consistent with the interactions between both domains suggested on the basis of cryoEM on uroplakins and modeling of CD81 [2,28]. However, an NMR study using soluble CD81 EC1 and EC2 could not detect any interaction, possibly because the full protein context is missing [23].

Whereas the TM region and the EC2 of tetraspanins have a rather defined structure, little or nothing is known about structural elements in the remainder of the protein (EC1, cytoplasmic tails and -loop). Thus, intramolecular interactions in the TM and EC2 region of tetraspanins support their overall structure and set the stage for intermolecular protein–protein interactions.

Tetraspanin–tetraspanin interactions: promiscuous and specific interactions underlie tetraspanin web formation

The first eras of tetraspanin research resulted in a model in which tetraspanins interact promiscuously (and mostly heterotypic) and extensively among each other to form the tetraspanin web [29]. This model, which was primarily based on immune precipitation studies, was recently challenged by studies based on chemical cross-linking and super-resolution microscopy, showing that several tetraspanins have a preference for specific homotypic interactions [30,31]. The observations on which both models are based suggest two modes of tetraspanin–tetraspanin interactions: promiscuous interactions and specific interactions.

An important mediator of promiscuous interactions is palmitoylation of cytoplasmic cysteine residues that are present in all known tetraspanins. The contribution of this reversible post-translational modification was

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first recognized in 2002 for tetraspanin–tetraspanin interactions between CD9 and CD151 [32,33], and was subsequently observed for CD9 [34], CD81 [35], CD82 [36] and CD63 [37]. From these studies, a model arises in which the tetraspanin web as a whole is supported by constitutive palmitoylation, whereas induced (de) palmitoylation can facilitate the plasticity needed to react to different stimuli [38]. In addition to palmitoylation, cholesterol also supports promiscuous tetraspanin interactions, probably in a palmitoylation-dependent manner [10,39]. Gangliosides are a third membrane component suggested to be involved in promiscuous tetraspanin–tetraspanin interactions as is proposed for CD82 and CD81 (Figure 2A) [40,41,24].

A potential tetraspanin domain involved in more specific interactions was identified when the CD81 EC2 crystal structure was found to be a dimer with an important role for the A- and, to a lesser extent, B- and E-helices in the interaction area [3]. Although the dimeric nature of the CD81 EC2 was confirmed in subsequent studies and the amino acids predicted to be important for dimerization could be confirmed in a random mutagenesis study, the same mutations had little or no effect on dimerization of the complete CD81 molecule [25,23]. Also arguing against the importance of this proposed dimerization domain in the complete protein is the fact that the position of the termini of both monomers hardly allows fitting of the TM domains into this homodimer model.

Another dimerization domain was identified using disulfide cross-linking which was used to map the homodimeric interface of CD9 to the TM1 and TM2 domains [17]. Although the authors showed that CD9 molecules interact among themselves via this domain, they did not test dimerization with other tetraspanins and thus did not determine the specificity for homotypic interactions. In fact, the high conservation of TM domains among tetraspanins makes it more likely that they are involved in promiscuous rather than specific tetraspanin–tetraspanin interactions.

A third domain that could account for specific homotypic interactions was identified when the D-loop of the CD81 EC2 was shown to be required for its recruitment into CD81 clusters on the plasma membrane and sufficient to recruit other tetraspanins into these clusters [42,43]. In the same study, a palmitoylation-deficient CD81 and an AB-helix deletion mutant were also tested, but these had a far less pronounced effect on CD81 recruitment into domains. Thus, the D-loop seems to be a good candidate for specific interactions, also given its high flexibility and high variety between tetraspanins (Figure 2A). A follow-up molecular modeling study suggests that the high flexibility of the D-loop in the otherwise relatively stiff EC2 domain allows tetraspanins to 'probe' the surrounding area for interaction partners [24]. The same model also suggests that when a specific interaction partner is found, more promiscuous binding factors, such as palmitoylation or gangliosides, further stabilize the interaction allowing formation of larger clusters. Whether the modeled interplay between promiscuous and specific interactions among tetraspanins indeed underlies formation of a tetraspanin web with predominantly homotypic tetraspanin–tetraspanin interactions remains to be confirmed in wet laboratory experiments, but this model is at least consistent with the current knowledge on these interactions.

Tetraspanin–partner interactions: adding functional partners to the tetraspanin web

The function and structure of the tetraspanin web is based on the ability of tetraspanins to form lateral associations with their partner proteins in order to influence their interaction capacity and function. Tetraspanins are known to interact with many partners including integrins (α3β1, α4β1 and α6β1), CD19, EWI-2, MHC complexes, CD4 and CD8 [44–48]. The adeptness of specific tetraspanins at binding selective partner proteins indicates that a high level of variability must exist between tetraspanins. Comparisons between sequences of different tetraspanins have pinpointed the large extracellular loop (EC2) of tetraspanins as the most variable domain [27].

Interactions between the EC2 and partners have been reported for numerous tetraspanins, one of the best characterized examples being the interaction between tetraspanin CD151 and integrins (Figure 2B). CD151 has been demonstrated to bind very strongly to the α3β1 integrin under stringent detergent conditions and is highly specific and stoichiometric [49]. Furthermore, this interaction was shown to be mediated by the variable head region of the EC2 domain since mutation of these residues left CD151 unable to bind either integrins or other tetraspanins [50]. Though the interaction between the head region of the EC2 domain of tetraspanins and their partners has long been proposed to be the paradigm, recent findings indicate that integrins are also able to strongly bind to the stalk region of the EC2, revealing the need for a more in-depth look at these regions with respect to tetraspanin–partner interactions [51]. The ability of tetraspanins to bind partners may

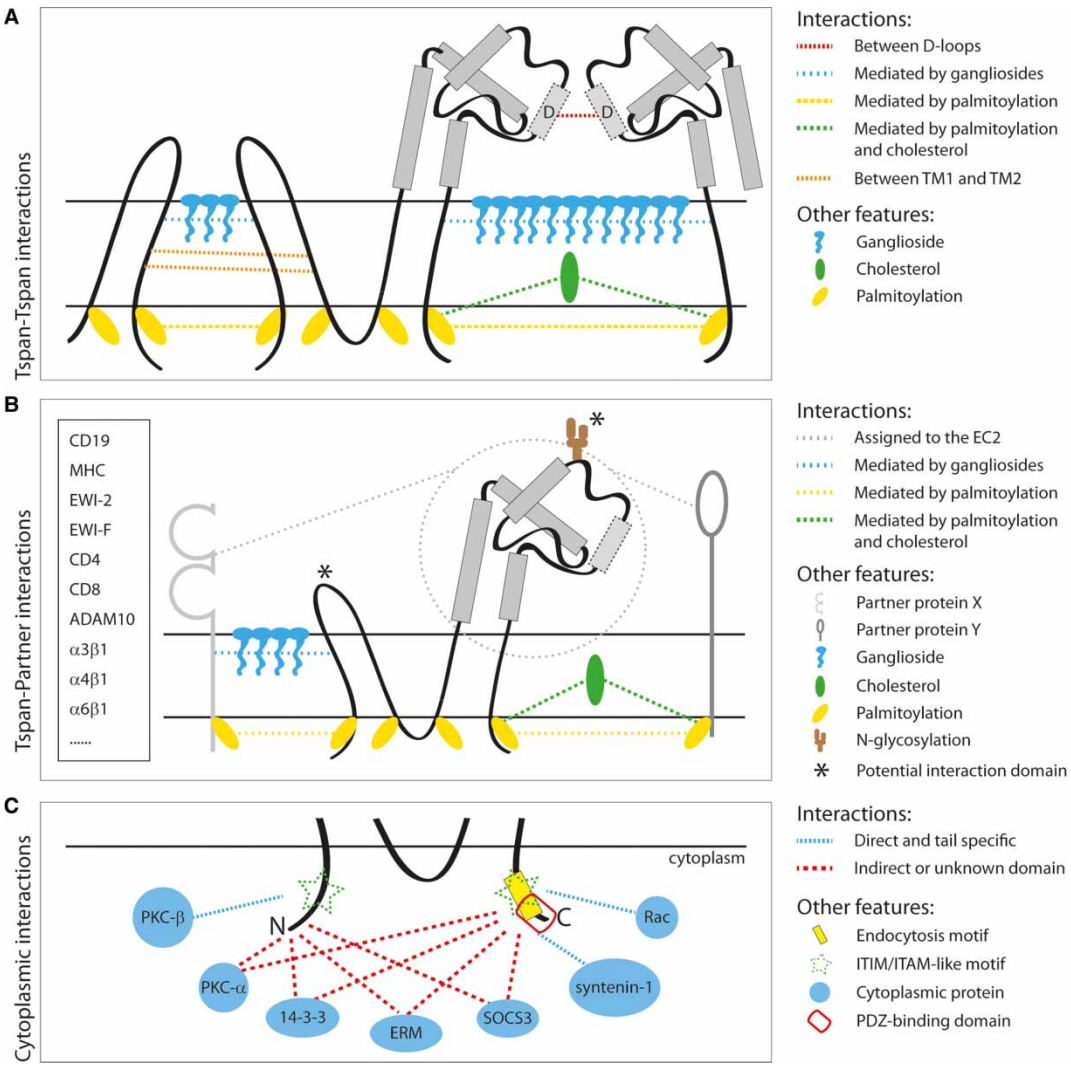

Figure 2. Intermolecular interactions of tetraspanins.

Models showing the different intermolecular interactions tetraspanins are capable of, which together shape the tetraspanin web. (**A**) Tetraspanin–tetraspanin interactions can be mediated by, and are probably a sum of, palmitoylation, cholesterol and gangliosides, and direct protein–protein interactions between TM1 and TM2 or the D-loops of different tetraspanins. The D-loops are suggested to mediate specificity to tetraspanin–tetraspanin interactions, whereas the other modes of interaction are likely of a more promiscuous nature. (**B**) Most tetraspanin–partner interactions are assigned to the EC2, although the precise interaction domain often remains elusive. A small selection of partner proteins binding the EC2 is boxed on the left. The role of the N-glycosylation sites that are present on the EC2 of many tetraspanins (the position in this figure is random) and the role of the EC1 in tetraspanin–partner interactions are a largely unexplored area. Tetraspanin–partner interactions can also be aided by palmitoylation, cholesterol and gangliosides. (**C**) Tetraspanin interactions with cytoplasmic proteins facilitate intracellular signaling events and are mediated by binding to the N- and C-terminal tails. Although many cytoplasmic proteins have been shown to interact with tetraspanins, direct binding to a specific tail has only been shown for a few of these proteins. The C-terminal tail of many tetraspanins contains an endocytosis motif and/or a PDZ-binding domain, and, arguably, some tetraspanins may harbor ITIM/ITAM-like domains which allow them to participate directly in signaling.

also be influenced by the conformation of the EC2. Recent findings by Zimmerman *et al.* indicate that the EC2 is capable of undergoing a conformational change depending on binding of cholesterol to the intramembrane pocket. The authors further suggest a relationship between EC2 conformation and CD19 interaction and expression, which indicates a possible mechanism by which the EC2 could modulate its binding to partner

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proteins [18]. Furthermore, recently, it has been shown that the EC2 of certain tetraspanins (tetraspaninC8 family members) can interact with the same partner (ADAM10) by distinct mechanisms and thereby differentially affect function of this partner protein [52].

The EC2 of most tetraspanins is N-glycosylated; however, the functional implications of this glycosylation remain largely unknown (Figure 2B) [53]. A few studies provide evidence that N-glycosylation is able to modulate the binding capacity of tetraspanins to partner proteins. For example, N-glycosylation of CD82 was shown to regulate adhesion and motility of cells through their interaction with α3 and α5 integrins [54]. Furthermore, the interaction between tetraspanin CD63 and CXCR4, leading to the down-regulation of this chemokine receptor, is regulated by *N*-linked glycosylation sites in CD63 [55]. Given the high prevalence and large size of the glycosylation found on tetraspanins, it seems likely that many of the interactions assigned to EC2 are affected, and maybe even mediated, by glycosylation of this domain. The possible role of glycosylation in tetraspanin function is supported by the reported variability in glycosylation of tetraspanins in cancer cell lines [56,57].

Although the EC2 is the most variable part of tetraspanins, the EC1 also displays quite some variability which may lend itself to mediation of tetraspanin–partner interactions. So far, no experimental evidence has been presented to support this notion, but phylogenomic analysis of intron/exon structure shows that the EC1 contains 46% of new intron insertions despite only accounting for 10.5% of the total protein length [58]. This appearance of evolutionary novelty in the EC1 loop (which is also found in the variable head region of the EC2) may support its possible role in defining the specificity of tetraspanins for their partner proteins.

So far, we have primarily focused on direct protein–protein interactions, but it should be noted that other membrane constituents also contribute to tetraspanin–partner interactions, including membrane cholesterol, gangliosides and palmitate moieties (Figure 2B). Cholesterol has been shown to directly interact with tetraspanins and has been proposed to play a role in the stabilization of tetraspanin interactions [39]. A role for palmitoylation in tetraspanin–partner interactions became apparent when palmitoylation-deficient β4 integrins were found to be less able to associate with tetraspanins including CD9, CD81 and CD63 [14]. It has also been shown that palmitoylation is required for the interaction of EWI-2 and EWI-2wint with CD81 and CD9 [59]. In addition, interactions aided by palmitoylation have functional consequences as demonstrated by Berditchevski et al. [32] who showed that palmitoylation of tetraspanins is essential for recruitment of partners to the tetraspanin web and modulates the function of these partners. Gangliosides are also suggested to be involved in tetraspanin–partner interactions as depletion of gangliosides was shown to affect the interaction between CD82 and its partners [41]. Furthermore, ganglioside GM3 has been found to interact with CD9, which is involved in down-regulation of tumor cell motility [60]. From the above, it can be concluded that cholesterol, gangliosides and palmitoylation are able to assist in both the stabilization and function of the tetraspanin web.

Taken together, tetraspanin function is defined by its ability to bind specific partner proteins and thereby influences cell function. Thus far, most studies suggest that the EC2 is primarily responsible for interactions between tetraspanins and partners, though the influence of other membrane constituents and protein modifications should not be overlooked in this process.

Cytosolic tetraspanin interactions: regulating intracellular signaling

Tetraspanins not only organize their partner proteins in the plasma membrane, but they are also often involved in protein organization in the cytoplasm beneath the tetraspanin web, thereby facilitating intracellular signaling processes. The idea that the tetraspanin web can function as a membrane scaffold for intracellular signaling arose when cytosolic protein kinase C (PKC) was found to associate with tetraspanins after PKC translocation to the plasma membrane upon PMA stimulus [61]. Subsequently, many associations between tetraspanins and signaling molecules have been described, e.g. with Ras, Rac, 14-3-3 and SOCS3, though the protein domains involved in these interactions often remained unstudied [13,62–64].

Downstream signaling processes initiated at the tetraspanin web can, in principle, be mediated through interactions between cytoplasmic signaling proteins and the intracellular loop or the N- and C-terminal tails of tetraspanins, but so far only a few examples of this have been shown (Figure 2C). The relatively short (4–40 amino acids for most tetraspanins) cytoplasmic extensions of tetraspanins are assumed to be flexible and show little conservation between different tetraspanin family members [5], which enables a range of tetraspanin-specific interaction partners. For many of the reported interactions, it is yet unclear whether these are direct or indirect due to limitations of the techniques used, but novel (microscopy) techniques have increased our ability

to distinguish direct from indirect interactions in live cells. Marked examples of direct interactions include the interaction between the N-terminal tail of CD53 and PKC-β which is important for B-cell receptor signaling (Zuidscherwoude and Dunlock, under revision), between the C-terminal tail of CD81 and Rac involved in the regulation of cell motility [64] and between the C-terminal tail of CD63 and the PDZ domain of syntenin-1-regulating endocytosis [65]. Since a PDZ-binding motif is present in the C-terminal tail of several other tetraspanins, cytosolic interactions mediated by PDZ domains are probably not restricted to CD63 [7]. The importance of intracellular tetraspanin interactions in signaling processes was nicely illustrated by Termini *et al.* [66] who showed that CD82-containing TEMs facilitate the formation of large PKC-α complexes on the inside of the plasma membrane which enable sustained PKC activation.

In addition to facilitating intracellular signaling through recruitment of cytoplasmic signaling proteins, tetraspanins can also play a more direct role in signaling as was demonstrated by Lapalombella *et al.* They showed that tyrosine residues in ITIM- and ITAM-like domains in the tails of CD37 are phosphorylated upon ligation with a CD37-directed antibody, which controls cell death signaling in B cells [67]. In line with this, CD37 deficiency affects plasma cell survival *in vivo* through coupling to the Akt kinase signaling pathway [68]. Although other tetraspanin family members may also harbor ITIM/ITAM-like (immune tyrosine-based inhibitory/activation motif) motifs, their functionality in signaling processes remains to be tested. A more common tyrosine-based signaling motif is the YXXΦ endocytosis motif found in the C-terminal tail of many tetraspanins (Figure 2C). This motif was shown to be a bona fide internalization signal for CD151, Tspan7 and CD82, and is assumed to be a general mechanism for endocytic trafficking of tetraspanins containing this motif [5,69]. In CD63, a C-terminal lysosomal targeting signal is present which orchestrates its lysosomal localization [70].

Apart from tetraspanin interactions with signaling molecules, interactions with the actin cytoskeleton have also long been suspected based on membrane fractionation experiments [71]. For tetraspanins CD9 and CD81, it is now known that this interaction is mediated by indirect binding with actin-linking ezrin–radixin–moesin (ERM) proteins via EWI proteins. Moreover, CD81 was shown to directly interact with ERM proteins via its C-terminal tail [72].

Taken together, the cytoplasmic interactions of tetraspanins are mediated by their N- and C-terminal tails. These interactions affect intracellular signaling pathways directly or indirectly, and are important for tetraspanin localization and interaction with the cytoskeleton.

Concluding remarks and future perspectives: toward tetraspanin web dynamics

In this review, we propose a new classification of tetraspanin interactions based on their function in the formation of the tetraspanin web rather than their resistance to different detergents. This classification not only correlates more directly with functionality, but also allows inclusion of important intramolecular interactions and interactions with non-membrane proteins. The different classes of functional tetraspanin interactions are not mutually exclusive. In fact, the unique capacity of tetraspanins to unify different types of interactions underlies their function as important regulators of membrane organization.

Whereas our view on the interactions and functions of the tetraspanin web is extending, its dynamics remain largely unexplored. Future research in this direction will be enabled by novel (super-resolution) microscopy techniques that allow visualization of changes in the size, shape, composition and distribution of the tetraspanin web in different cell types. We strongly believe that a thorough understanding of tetraspanin interactions is key to further uncovering tetraspanin dynamics and its important role in cell biology of health and disease.

Abbreviations

EC1, extracellular loop 1; EC2, extracellular loop 2; ERM, ezrin–radixin–moesin; ITAM, immune tyrosine-based activation motif; ITIM, immune tyrosine-based inhibitory motif; PKC, protein kinase C; TEM, tetraspanin-enriched microdomain; TM, transmembrane.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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