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Review

Functional competition within a membrane: Lipid recognition *vs.* transmembrane helix oligomerization☆

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ABSTRACT

Binding of specific lipids to large, polytopic membrane proteins is well described, and it is clear that such lipids are crucial for protein stability and activity. In contrast, binding of defined lipid species to individual transmembrane helices and regulation of transmembrane helix monomer–oligomer equilibria by binding of distinct lipids is a concept, which has emerged only lately. Lipids bind to single-span membrane proteins, both in the juxtamembrane region as well as in the hydrophobic membrane core. While some interactions counteract transmembrane helix oligomerization, in other cases lipid binding appears to enhance oligomerization. As reversible oligomerization is involved in activation of many membrane proteins, binding of defined lipids to single-span transmembrane proteins might be a mechanism to regulate and/or fine-tune the protein activity. But how could lipid binding trigger the activity of a protein? How can binding of a single lipid molecule to a transmembrane helix affect the structure of a transmembrane helix oligomer, and consequently its signaling state? These questions are discussed in the present article based on recent results obtained with simple, single-span transmembrane proteins. This article is part of a Special Issue entitled: Lipid–protein interactions.

Contents

1. Dimerization of TM helices regulates cellular functions

Folding of large, polytopic transmembrane (TM) proteins involves interactions of multiple TM helices, and thus individual TM helix–helix interactions can affect or even dictate the assembly of large protein complexes [1–4]. In fact, altered interaction propensities of individual TM helices might be linked to various diseases, due to destabilization or misfolding of polytopic TM proteins [4–6]. However, almost half of the whole human TM proteome consists of single-span TM proteins [7,8]. Single-spanning membrane proteins (MPs) mediate a wide range of cellular processes, including cell–cell adhesion (integrins) [9,10], immune recognition (major histocompatibility complex, MHC) [11] and signal transduction (*e.g.*, receptor

Abbreviations: TM, transmembrane; MP, membrane protein; GpA, glycophorin A; PIP, phosphatidylinositol phosphate; PI, phosphatidylinositide; RTK, receptor tyrosine kinase; MHC, major histocompatibility complex; PG, phosphatidyl glycerol; PS, phosphatidyl serine; PLC, phospholipase C; PH, pleckstrin homology; NMR, nuclear magnetic resonance; COP, coat protein complex; APP, amyloid precursor protein; ErbB, epidermal growth factor receptor; CRAC, cholesterol recognition amino acid consensus; CARC, inversed cholesterol recognition amino acid consensus; Kir, inwardly rectifying potassium channel; ER, endoplasmic reticulum; GOLD, Golgi dynamic; HIV, human immunodeficiency virus; SBD, sphingolipid-binding domain; SM, sphingomyelin; GPCR, G-protein coupled receptor; SNARE, soluble *N-ethylmaleimide sensitive factor* attachment protein receptor; CCM, cholesterol consensus motif

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tyrosine kinases, RTKs) [12], and contacts between individual bitopic MPs are common [13,14]. Importantly, the TM helices that anchor MPs in the membrane are often critically involved in oligomerization of the full-length MPs. While strongly associating single-span TM helices are thought to form stable membrane-inserted protein–protein complexes, modestly strong interacting TM helices exist in a dynamic equilibrium of the free monomers and the associated oligomers. Reversible oligomerization of individual TM helices can trigger and regulate signaling processes at and across cellular membranes. *E.g.,* while dimerization of the various integrin α- and β-subunits is not completely understood, the respective TM domains are most likely crucially involved in integrin dimerization, and it has been shown that integrin TM domain interactions trigger integrin functions [15–19]. The immune active MHC class II complex is formed by an α/β-heterodimer and invariant chain proteins. Recent results also indicate that here TM helix–helix contacts are crucial for formation of the MHC II complex [20]. RTKs form dimers or even higher-ordered multimeric complexes, and a plethora of data has demonstrated in recent years that dimerization and activation of RTK-family members are mediated by the single TM helix [21–26]. In line with this, the isolated single-span TM domains of all human RTKs have been shown to have an intrinsic propensity to interact, and thus oligomerization of RTK TM helices appears to be common [27]. In the case of ErbB (HER) proteins, probably the best characterized RTK-family members, defined adjustments of the TM helix dimer structure appear to be involved in signaling [21,28]. A recent analysis of the human single-span TM proteome has revealed that the isolated TM helices of many single-span TM proteins have an intrinsic propensity to form higher ordered oligomeric structures [14], and thus oligomerization of single-span TM proteins appears to be the rule rather than the exception.

Molecular forces driving interactions of single- and multi-span TM proteins within the membrane include Van der Waals interactions, resulting from close packing of interacting helices, hydrogen bonding, as well as ionic and aromatic interactions [5,29–31]. That formation of tightly packed, homo-oligomeric helix bundles is driven by sequence-specific interaction of TM helices was demonstrated more than 25 years ago for the TM domain of the human glycophorin A (GpA) protein [32], a membrane integral protein located in the red blood cell plasma membrane. Later, seven amino acids of the LIxxGVxxGVxxT-motif were identified in a mutational study to be involved in dimerization [33–35]. The GxxxG-core of the GpA interaction motif turned out to be highly overrepresented in TM proteins and still represents the most significant motif in interacting TM helices identified thus far [36,37]. Besides this, several motifs mediating oligomerization of TM domains have been identified, including Ser and/or Thr-containing motifs [38,39], motifs containing aromatic residues [40,41] or residues with carboxamide side chains [42–47], as well as the QxxS-motif [48,49]. More than one dozen high-resolution structures of simple TM helix oligomers have been published in recent years, revealing defined helix–helix contact interfaces. However, often no defined interaction motifs have been identified, and two TM helices interact by forming complementary surfaces, which allow close helix packing, as summarized recently in Cymer *et al.* [30]. However, since reversible interactions of TM helices might be involved in inhibition or activation of the full-length proteins, TM helix oligomerization has to be regulated to avoid constitutive activation or inhibition of the proteins. Formation and stability of TM helix bundles are not only defined by the specific amino acid context, but also by the composition of the intimate lipid environment, as well as by the overall physico-chemical properties of the membrane. MPs communicate with the lipid environment and thereby the association and activity of MPs might be manipulated and/or triggered.

2. Lipids interact with membrane proteins

Eukaryotic membranes are composed of diverse phospholipids with different head groups and acyl chain lengths as well as cholesterol [50]. It is not finally resolved yet why membrane lipids have different acyl chain lengths. Possibly, it is important for grouping proteins and lipids with similar hydrophobic thicknesses, as hydrophobic regions of TM domains also differ in their length in MPs. In fact, based on the OMP database [51], the hydrophobic thickness of dimeric single-span human TM proteins found in the human plasma membrane varies between 30 and 36 Å, which strongly indicates that the thickness of the lipid bilayer locally adjusts to completely mask the hydrophobic region. Hydrophobic mismatch conditions can result in protein aggregation within lipid bilayer environments [52–56].

Besides the hydrophobic thickness of the membrane, the lateral pressure profile within the acyl chain region as well as the distribution of lipid head group charges at a protein–lipid interface control interactions of MPs with lipids [30,57–59]. In general, lipid binding to a MP can be stabilized by electrostatic and hydrophobic interactions between the lipid head groups and amino acid residues and additionally by a large number of hydrophobic interactions between the hydrophobic lipid tails and TM moieties of the protein (Fig. 1).

Based on the residence time of a particular lipid at the lipid–MP interface, three types of interactions of lipids with MPs might be distinguished (Fig. 2) [60]. Lipids, which diffuse rapidly within the bilayer plane and show a low residence time at the protein–lipid interface, so-called bulk lipids, do not directly affect the structure and/or function of MPs. The bulk lipid phase represents the total lipid volume of the membrane and determines its global characteristics, such as the membrane fluidity, the lateral pressure, the bilayer thickness or the membrane surface charge. When the polar lipid head group interacts with a MP or when hydrophobic matching between the lipids and the TM domain of the MP is crucial, the residence time of the lipids might significantly increase and a shell of annular lipids is formed around the MP. The composition of this annular lipid shell is determined by the local architecture of the protein. In the annular lipid shell, which is composed of around 50–100 lipids and which is not necessarily homogeneous [61], the specific characteristics of the lipids can strongly affect the structure and function of a MP [62,63].

If the interaction of lipids and MPs is even stronger, the so-called non-annular surface lipids will bind specifically and tightly to MPs, typically in cavities and clefts of hydrophobic binding pockets [64]. Non-annular lipids often remain bound to MPs, even if the MPs were purified and crystallized in detergent [65,66]. Especially in larger protein complexes, non-annular lipids fill the crevices between adjacent monomers or subunits and thereby mediate protein complex formation. These lipids seem to play an important role in the structural stability of MPs, and tightly bound lipids can be essential for the activity of MPs [67].

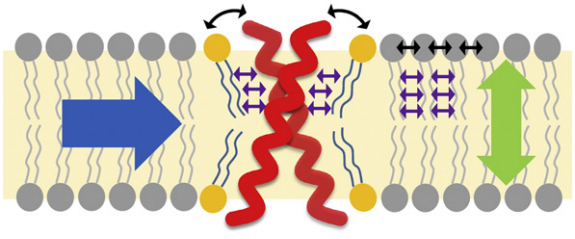

Fig. 1. How the lipid environment can affect TM protein structures. Non-annular lipids (orange) bind specifically at the surface of TM proteins *via* salt bridges between charged lipid head groups and charged residues at the membrane-water interface (black arrows). Hydrophobic, Van der Waals and weak dipolar interactions might additionally be involved in lipid binding. Van der Waals interactions between the acyl chain and hydrophobic amino acids further contribute to tight lipid binding (purple arrows). Bulk lipids define the global membrane environment of TM proteins and affect membrane protein folding *via* membrane properties, such as the hydrophobic thickness (green arrow) and the lateral membrane pressure profile (blue arrow). The geometry of the lipids (bilayer-forming *vs.* non-bilayer-forming) as well as electrostatic interactions between the lipid head groups (black arrows) and packing of the lipid acyl chains determine the global membrane properties.

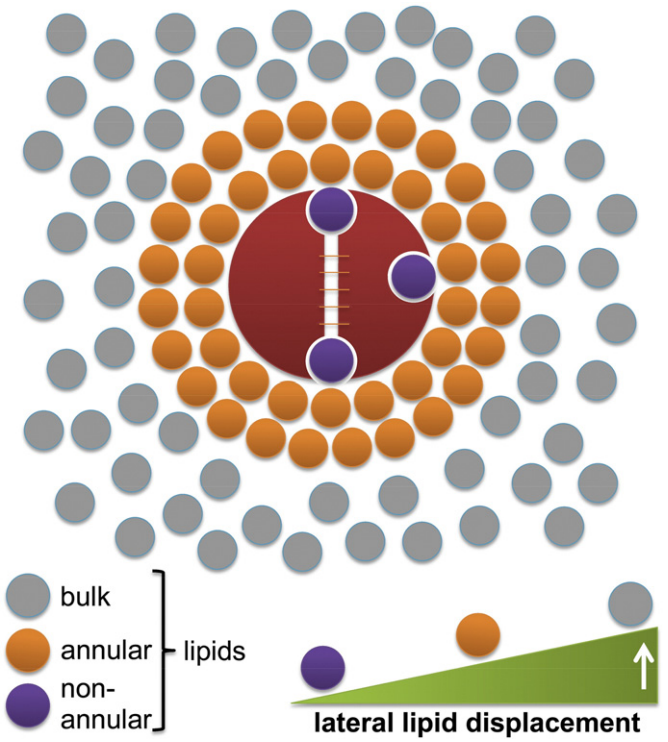

Fig. 2. Intramembrane protein–lipid interactions — a top view on the membrane. A membrane protein dimer stabilized by tightly bound non-annular lipids. Non-annular lipids fit into cavities at the protein surface, and these lipids are often found to be still bound in isolated proteins. A belt of annular lipids define the intimate environment of a membrane protein. While the structure and size of this lipid belt varies, it was suggested that a protein is typically surrounded by 50–100 annular lipids [61]. Annular lipids have higher exchange rates at the membrane protein than non-annular lipids, but the diffusion rate of the annular lipids is significantly reduced compared to the bulk lipid phase. Lipids with low degree of interaction with the TM protein are considered to be "bulk" lipids, which have high lateral displacement and diffusion rates.

This is *e.g.,* observed in the case of the KscA potassium channel, which is only active when negatively charged lipids are bound [68–70], and ADP/ATP carriers require binding of cardiolipins (compare Fig. 3) for activity [71,72]. More examples and detailed information on how non-annular lipids affect MPs' activities can be found in Lee *et al.* [62].

Cholesterol binding to MPs has been studied to a great extent in recent years. A cholesterol-binding motif was initially identified in the peripheral-type benzodiazepine receptor [73] (Table 1). The CRAC (*C*holesterol *R*ecognition *A*mino acid *C*onsensus) motif (L/V-$(x)_{1-5}$-Y-$(x)_{1-5}$-R/K), where x represents an arbitrary amino acid, consists of hydrophobic, aromatic and positively charged amino acids. Later, the reversed motif (CARC-motif, K/R-$(x)_{1-5}$-Y-$(x)_{1-5}$-L/V) (Table 1) was postulated to be important for cholesterol binding [74]. In general, binding of cholesterol appears to require the presence of a polar amino acid that is able to hydrogen bond to the 3β-OH group of cholesterol (compare Fig. 3), as well as small hydrophobic as well as aromatic amino acids that are involved in hydrophobic and π–π stacking interactions at the lipid–protein interface [75]. However, the interaction of CRAC and CARC motifs with cholesterol remains unclear, and the currently available MP X-ray structures do not indicate that any distinct amino acid motif mediates cholesterol binding to MPs [67]. Furthermore, the CRAC motif has been identified more than 5000 times in the proteome (2100 proteins) of a cholesterol-free bacterium [76], and thus the prediction value of cholesterol-binding sites, using these motifs, appears to be very low. Furthermore, cholesterol, one of the best studied lipids in biochemical and medical research, has a dramatically different structure than typical bilayer-forming diacyl phospholipids (Fig. 3). Thus, cholesterol binding might be rather specific.

2.1. Binding to negatively charged lipid head groups can control TM peptide oligomerization and clustering

The impact of global bilayer properties on the oligomerization of TM helices has already been analyzed to some extent, and single-span TM helices frequently serve as manageable models to reveal the impact of the lipid bilayer on a MP structure. While binding of specific non-annular lipids to polytopic TM proteins has been identified and analyzed to some degree in the past, recent work has also identified lipid-recognition by single-span TM helices, and lipid binding appears to severely affect protein folding as well as the cellular functions of the proteins (as further discussed below).

The GpA TM helix dimer has for a long time served as a paradigm in studies, aiming at identifying sequence determinants in a TM helix–helix interaction. Several recent *in vitro* studies have shown that the detergent environment can severely affect TM helix dimerization propensities of GpA and other dimerizing TM helices [77–85]. Global lipid bilayer properties, such as the order of the lipid acyl chains or the membrane thickness, also affect the structure of the GpA TM helix in membranes [86–88]. Furthermore, in model membrane systems, the anionic lipids phosphatidylglycerol (PG) and phosphatidylserine (PS) (compare Fig. 3) severely destabilize the GpA TM helix dimer [89]. The negatively charged lipid head groups appear to specifically bind at the juxtamembrane region to a stretch of basic amino acids, which follow the C-terminus of the GpA TM helix [89]. Binding of the negatively charged lipids destabilized the GpA helix dimer, although it is currently unclear how the TM helix dimer structure is weakened. How is the signal "bound lipid" transferred from the juxtamembrane region to the TM helix–helix interface, resulting in TM helix dimer destabilization? Is this deleterious effect merely based on the negative net charges of the lipids but not on the acyl chains? And, if solely the negative charge matters, how do even more negatively charged lipids, such as phosphatidylinositol phosphates, affect the structure of oligomeric single-span MPs after binding?

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is the most abundant PI in mammalian plasma membranes, with about 1% of the total lipid located in the inner leaflet of the membrane [90,91]. PIPs are lipids with an inositol head group conjugated with three phosphate groups (Fig. 3). The phosphate at the first carbon atom is esterified with glycerol that carries two fatty acid residues. PIP_2 with its two phosphate groups at carbon atoms four and five is *e.g.,* a substrate of phospholipase C (PLC), controlling downstream signaling cascades [92,93]. Furthermore, PIP_2, as well as its phosphorylated form PIP_3, can also directly act as a docking lipid for enzymes, thereby recruiting proteins to the plasma membrane [91,94]. PIP_2 electrostatically interacts *via* its negatively charged phosphate groups with non-contiguous basic residue-rich clusters at proteins [91,95]. The PIP phosphates at positions four and five form several hydrogen bonds with residues in the PLC pleckstrin homology (PH) domain, and especially electrostatic interactions with two lysine residues fasten the protein at the membrane surface [96,97]. However, >250 other identified PH domains only weakly interact with inositides, rendering lipid binding exclusively to the PLC PH domain unlikely. Besides the PH domain, other PIP_2 binding domains exist in soluble proteins, as discussed in greater detail in recent reviews [91,95,98]. However, are PIP_2 binding domains also present in integral MPs? In fact, the K^+ channel Kir2.1 requires binding of multiple PIP_2-molecules for channel activity. Here, PIP_2 electrostatically interacts with three independent sites at the channels' C-terminus, thereby stabilizing an active channel conformation [99]. While the TM domain appears to bind any diacylglycerol with a 1′ phosphate, the juxtamembrane region specifically interacts only with the PIP_2 head group and thereby defines the lipid-binding specificity. Importantly, while PIP_2-binding does not change the tetrameric assembly of the related Kir2.2 channel, lipid binding induces a conformational change in a flexible linker region, which results in reorganization of the entire channel structure and finally in channel activation [100].

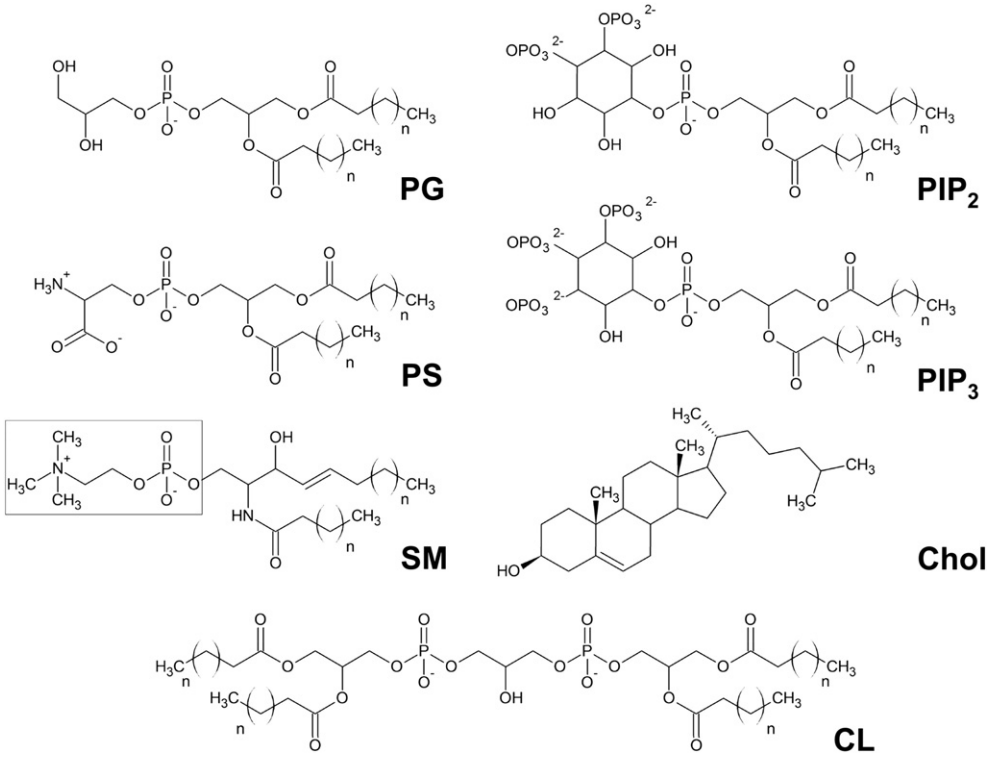

Fig. 3. Chemical structures of lipids. Depicted are the lipid species discussed in this article. PG: phosphatidylglycerol, PS: phosphatidylserine, SM: sphingomyelin, PIP_2: phosphatidylinositol 4,5-bisphosphate, PIP_3: phosphatidylinositol-3,4,5-trisphosphate, Chol: cholesterol, CL: cardiolipin. Typical n-values of the discussed phospholipids vary between 12 and 18. In the case of the SM C18:0 sphingomyelin species discussed in the text, the fatty acid chain carries 18 carbon atoms. Not shown is ceramide, which is SM without the phosphocholine head group (boxed).

PIP_2 might form clusters in eukaryotic plasma membranes, and several studies showed a co-localization of syntaxin 1A with such PIP_2 clusters, indicating that PIP_2 is required for syntaxin clustering [101–103]. The membrane target SNARE (tSNARE) syntaxin 1A is composed of a N-terminal three-helix bundle H_{abc}, an amphipathic helix H_3 that interacts with other SNARE proteins to form a fusion complex (or the H_{abc} domain), and a C-terminal TM domain [104–107]. Interaction of PIP_2 with the single TM helix of syntaxin 1A leads to sequestration of the protein and the lipid [102]. Even though it is much less abundant than PIP_2 in cellular membranes, PIP_3 is also important for syntaxin 1A clustering and the function of the SNAP–SNARE complex [94]. The interaction of syntaxin 1A with PIP lipids is mediated by a stretch of basic amino acids. The critical residues are directly adjacent to the TM helix and are in contact with the lipid head groups [108,109]. The positively charged residues of the ^{260}KARRKK^{265} amino acid motif interact with PIP_2 (Fig. 4), and a strong reduction of the lipid–protein interaction was observed when Lys^{264} and/or Lys^{265} were mutated to Ala [102]. Mutation of the wt syntaxin 1A sequence also led to reduced vesicle fusion in cells, which additionally demonstrates the *in vivo* importance of the basic amino acids and of PIP binding [110]. PIP_3 binds even more efficiently to syntaxin 1A and can replace PIP_2 at the interaction site, also mediated by electrostatic interactions with the above-mentioned stretch of positively charged amino acids [94]. Recently, PIP_3 has been identified as an inducer of syntaxin 1A clustering in cellular membranes [94], and the concomitant mutation of Lys^{264} and Lys^{265} abolished PIP_3-syntaxin 1A clustering. These data indicate that mainly electrostatic interactions stabilize the binding of PIPs to syntaxin 1A, due to the strong negative net charge of the lipid head groups. As also observed in the case of the Kir2 K^+ channels, no further structural prerequisites for PIP binding to syntaxin 1A have been described yet. Additionally, as PS, with its one net negative charge, does not induce syntaxin 1A clustering, even in the presence of 20% PS [94], not only the negative head group charge but also the chemistry of the lipid head group might matter. Furthermore, the entropy cost for binding two or three lipids *via* electrostatic interactions is much higher than binding a single lipid with multiple charges, so that binding of PIP_3, with its four negative charges, might be preferred over PIP_2 or PS. PIPs mediate syntaxin 1A clustering, and additionally

Table 1
Lipid binding motifs identified in membrane integrated proteins.

Motif	Bound lipid	aa sequence	First identified	Ref.
CRAC	Cholesterol	L/V-$(x)_{1-5}$-Y-$(x)_{1-5}$-K/R	P-type benzodiazepine receptor	[73,185]
CARC	Cholesterol	K/R-$(x)_{1-5}$-Y/F-$(x)_{1-5}$-L/V	Nicotinic acetylcholine receptor	[74,185]
CCM	Cholesterol	Formed by helices 1–4[a] [4.39–4.43 (R,K)]–[4.50 (W,Y)]–[4.46 (I,V,L)]–[2.41 (F,Y)]	β_2-adrenergic receptor	[185,186]
Tilted peptides	Cholesterol	ExxxxNxGxxxGxxxGG	C99	[146,185,187]
SBD	Glycosphingolipid	Loop: aromatic AAs + basic AA in proximity	HIV gp120, APP	[124,128]
	Sphingomyelin C18:0	VxxTLxxIY	p24	[123,130]
PIP binding motif	Phosphatidylinositol-phosphates	KARRKK	Syntaxin 1A	[94,102,188]

For further information and examples see reviews [64,189].
[a] Numbering based on the Ballesteros–Weinstein. x = apolar aa-residue.

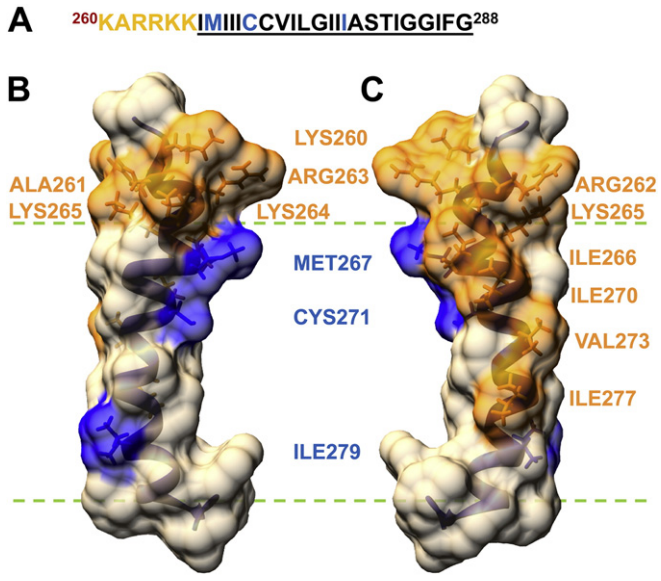

Fig. 4. The syntaxin 1A transmembrane helix. (A) Amino acid sequence of the syntaxin 1A TM region. The TM domain is underlined. (B) Surface structure of the syntaxin 1A helix. (C) Rotated syntaxin structure (~180°). (B, C): PDB ID: 2M8R. Residues involved in lipid (PIP) binding are highlighted in orange, whereas residues triggering dimerization of the TM helices are depicted in blue.

the syntaxin TM domain homodimerizes [111,112]. Critical residues mediating homodimerization of syntaxin and heterodimerization with its natural interaction partner synaptobrevin II are Met^{267}, Cys^{271} and Ile^{279} [112] (Fig. 4). Looking at the NMR structure of syntaxin 1A, the β-branched Ile residues 266, 270, 277 (possibly also Val^{273}) form a smooth and very hydrophobic surface at one side of the syntaxin 1A TM helix (Fig. 4). These residues are most likely not involved in dimerization of syntaxin 1A, and thus they might additionally stabilize PIP_2 binding *via* hydrophobic interactions between the lipid acyl chains and the hydrophobic TM surface. However, such an assumption has to be tested in future experiments.

2.2. Sphingomyelin binding to the transmembrane helix triggers oligomerization of the COP I machinery protein p24

Formation of COP I and COP II complexes is a crucial step in the transport of proteins between the endoplasmic reticulum (ER) and the Golgi apparatus in eukaryotic cells. COP I is involved in the anterograde vesicle transport from the ER to the Golgi, whereas COP II vesicles are involved in the retrograde transport from the Golgi to the ER [113,114]. For traveling between these compartments, both pathways depend on immobilization of coat complexes at the vesicular surface. Coat complex formation at the vesicle surface is mastered by a type-I TM protein with a mass of ~24 kDa, hence the name p24. Potential roles for p24 proteins in cargo reception and coat recruitment are discussed in Strating *et al.* [115]. The p24 protein family can be subdivided into four subfamilies (α, β, γ, δ) [116]. However, the bewildering variety of names of several members of the p24 family makes it difficult to identify the protein of interest [115]. The p24 protein we discuss here is the protein $p24\beta_1$, or TMED2, or p24 or p24a and will further just be called p24.

All p24 proteins share a similar structural arrangement. A large globular N-terminal GOLD (Golgi dynamic) domain [117] is located at the luminal side of the membrane. The exact function of this domain is enigmatic but it co-occurs with lipid-, sterol- or fatty acid-binding domains, such as PH, Sec14p, FYVE and RUN [117]. The GOLD domain is followed by an undefined coiled-coil region, a TM α-helix and a short C-terminal cytoplasmic tail, which is involved in COP I and COP II coat complex binding [115,118]. p24 proteins are known to form homo- and heterodimers, depending on their localization [119]. An involvement of the coiled-coil [120] and the cytoplasmic region in p24 dimerization is discussed [121,122], albeit recent results suggest that TM helix–helix interactions mediate dimerization of p24, triggered by binding of a sphingolipid [123].

Sphingolipid-binding motifs were initially identified in HIV-1 virus and amyloid proteins (Table 1) [124–127]. The identified motif is part of a hairpin structure of the gp120 protein at the surface of HIV-1 and Alzheimer-β-amyloid peptides. The aromatic residues Tyr, Trp and Phe are part of the sphingolipid-binding domain (SBD) and the glycosphingolipids are mainly bound *via* π-stacking and electrostatic interactions with the sugar head groups. These interactions are accompanied by structural rearrangements of both binding partners [128]. The interaction motif, which has also been identified in the serotonin receptor family and can be predicted in several other MPs [128,129], binds glycosylated sphingolipid species restrictively.

p24 selectively binds a single sphingomyelin (SM) species, SM C18:0, but not ceramide (lacking choline head group) or phosphocholine analogs (Fig. 3) [123]. Further analyses revealed a remarkable preference of p24 to bind C18:0 SM, and shorter or longer chain length SMs appear to not interact with the p24 TM domain [123]. Thus, p24 preferentially binds sphingomyelins and both the head group, as well as the hydrophobic acyl chain regions of the lipids are important. As the head group of phosphatidylcholine and SM is identical, the hydrophobic acyl chain region is supposed to determine the specific binding of SM derivatives to the p24 TM helix. To identify amino acids involved in lipid binding, each residue of the p24 TM domain was mutated to Ala and SM binding was analyzed. Based on this analysis, a VxxTLxxIY amino acid motif in the TM helix determines SM binding to p24 (Table 1). Molecular modeling has indicated that SM C18:0 fits perfectly into a cavity formed by the residues Val^{13}, Thr^{16} and Leu^{17} (Fig. 5) [123]. Mutation of a single residue in this cavity to an amino acid with a bulky side chain in fact completely abolished SM binding [123]. However, it was suggested that the lipid head group too is important for binding since the structurally similar ceramide did not interact with p24. Unfortunately, the described binding motif only covers the TM helix. It is very likely that electrostatic interactions between the protein and the sphingolipid head group also play a role in lipid binding, as *e.g.,* described above concerning syntaxin 1A. In the case of p24, only a few amino acids are available for interactions, as the C-terminus, attached to the sphingolipid-binding site, is very short

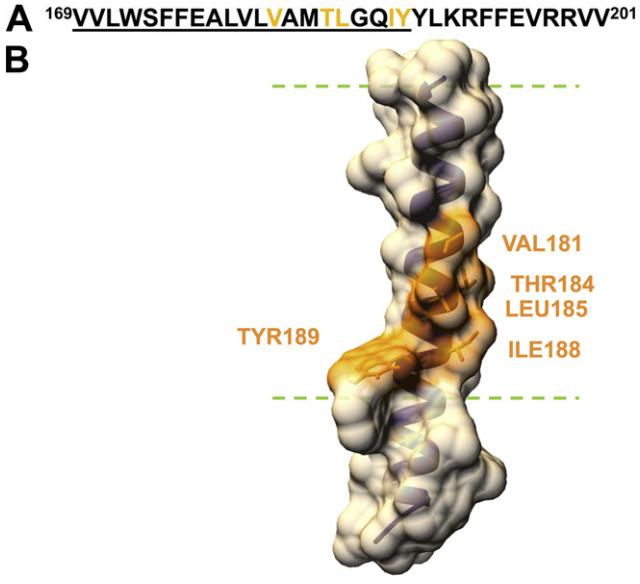

Fig. 5. The p24 TM helix. (A) Amino acid sequence of the p24 TM region. The TM domain is underlined. (B) Structure of the p24 TM helix. The structure was modeled using the PEP-FOLD Peptide Structure Prediction Server [190–192]. Residues involved in lipid (SM18:0) binding are highlighted in orange.

and also involved in COP vesicle contacts. It is worth mentioning that sphingolipids also bind to G-protein-coupled receptors (GPCRs), mediated by an amino acid motif, similar to the p24 sphingolipid-binding motif, and sphingolipid binding to a single TM helix of the receptor might stabilize different GPCR conformations [130].

As mentioned, p24 is able to form homo- or hetero-dimers with other proteins of the p24 family, which is a crucial step in COP vesicle formation. However, dimerization of p24 appears to be linked to sphingolipid binding. In SM C18:0 containing liposomes, p24 homodimerization is significantly increased, whereas a SM-binding deficient mutant has a decreased dimerization propensity, even in the presence of SM C18:0. Unfortunately, the dimerization interface in the p24 TM helix has not been described yet, although a molecular dynamics simulation study suggests a rather polar dimerization interface, which does not overlap with the sphingomyelin binding site [123].

It is suggested that the dimeric p24 family proteins immobilize the COP complex at the membrane surface. However, the SM concentrations, which could severely modulate dimerization of p24, significantly differ from the ER to the Golgi and the plasma membrane. Thus, the sphingolipid concentration within a given organelle membrane might directly influence the secretory pathway by triggering the oligomeric state of p24. However, the heterogeneous distribution of SM within a single membrane further complicates such an interpretation, as sphingolipids and cholesterol can form specific lipid microdomains in cellular membranes [131–137].

2.3. C99, the β-secretase cleavage product of the amyloid precursor protein APP specifically binds cholesterol

The TM protein C99, also known as β-CTF, is a key protein in the amyloidogenic pathway. It is associated with the release of amyloid β-peptides and therefore represents a key protein in the development of Alzheimer's disease [138–141]. C99 is produced by the amyloidogenic cleavage of the full-length amyloid precursor protein APP, catalyzed by the β-secretase [141]. This cleavage generates the 99-amino-acid-long single-spanning TM protein C99, which is then cleaved inside the membrane by the γ-secretase to release amyloid-β peptides [142,143]. The structure of the monomeric as well as of the dimeric C99 has been determined by NMR in micellar solutions [144–146] (Fig. 6). An extracellular N-terminus is followed by a surface-attached helix (N-helix) (residues 688–694) and a flexible loop (N-loop) (residues 695–699). The highly curved TM helix (residues 700–723) is kinked close to the center of the micelle, near Gly^{708} and Gly^{709}. This might be related to the processive cleavage by the γ-secretase, as flexibility allows the helix to adopt to the sluice-like active site of the protease [146–149]. Importantly, the proteolytic efficiency is enhanced 2–4 fold in the presence of cholesterol [150].

In recent studies a cholesterol-binding site and a homodimerization interface at C99 were defined [146,151–153]. Homodimerization and cholesterol binding were found to compete, as both involve the glycine zipper motif $G^{700}xxxG^{704}xxxG^{708}G^{709}$ [151] (Fig. 6A). Especially the GxxxG-motifs were expected to promote tight packing of two adjacent C99 TM α-helices, resulting in Van der Waals packing interactions and potentially in formation of C_α hydrogen bonds, as demonstrated for other TM proteins [3,103,154,155]. Indeed, both APP as well as the C99 fragment were found to dimerize, and dimerization is mediated by the GxxxG-motif [110,144,145,156–159]. The isolated C99 TM helix was found to oligomerize strongly in a bacterial membrane when measured with the ToxR-system, and oligomerization was impaired when critical Gly residues of a GxxxG-motif were replaced [110]. Based on the current available data, the β-secretase cleavage product of APP, C99, forms a stable dimer, stabilized by the glycine zipper motif located in the TM domain.

However, the physiological relevance of C99 dimerization is still a matter of debate [151]. In fact, the equilibrium dissociation constant for the dimer was determined to be in the range of 0.5 mol%, indicating

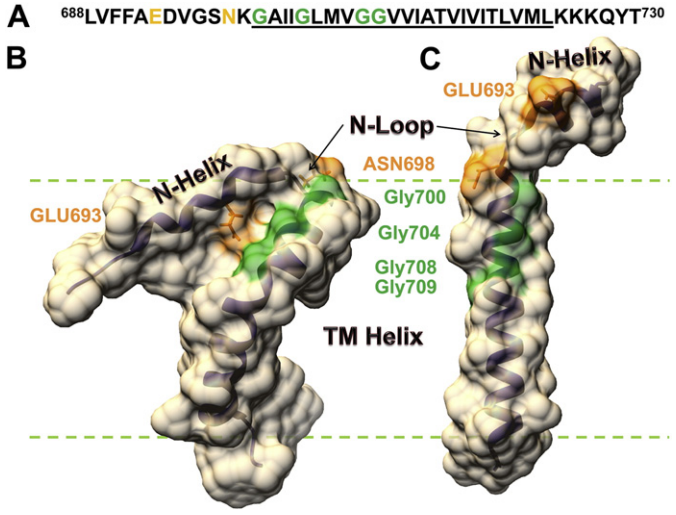

Fig. 6. The TM protein C99. (A) Amino acid sequence of the C99 protein. The TM domain is underlined. (B) C99 structure determined in lyso-myristoylphosphatidylglycerol detergent micelles by nuclear magnetic resonance (NMR) spectroscopy at pH 6.5 (PDB ID: 2LP1). (C). C99 structure determined in dodecylphosphocholine detergent micelles by NMR spectroscopy at pH 4.3–5.3 (PDB ID: 2LLM). In both structures the N-helix and N-loop are labeled, which can rotate freely as their relative position to the TM helix is random in different calculated conformers. The TM helix appears to be more kinked in the 2LP1 structure (B). Residues involved in lipid (cholesterol) binding are highlighted in orange. Amino acids involved in both lipid binding and TM helix dimerization are depicted in green.

that the protein appears to be mainly monomeric at a C99-to-lipid ratio of 1:200. While the exact C99 concentration has not been quantified in eukaryotic cells, the concentration of the amyloid precursor protein APP in neuronal membranes is in the range of 10^{-3}–10^{-4} mol% [159]. Therefore, C99 might be mostly monomeric under physiological conditions, while it cannot be ruled out that C99 exceeds the concentration of 0.5 mol% in heterogenic membranes and defined lipid domains, where it possibly forms dimers. Recently, the protein and lipid composition of a synaptic vesicle has been determined [160], and here on average about 600 TM domains together with ~7000 phospholipids and ~5000 cholesterol molecules form the vesicle membrane. About one quarter of the membrane volume is represented by the TM domains, and as annular lipids bind more tightly to the TM domains, only a minor fraction of the lipids is expected to be free. At a TM helix-to-lipid ratio of ~1:20, as determined in this study, a significant fraction of the C99 TM domain might be dimeric.

Cholesterol, which is crucial for the formation of lipid domains [161], is suspected to be involved in the development of Alzheimer's disease, as neuronal cholesterol levels increase the production of amyloid-β peptides. Both APP as well as solely the C99 fragment can specifically bind cholesterol, and consequently the C99 TM region is responsible for cholesterol attraction, while the N-terminal C99 extra-membrane domain contributes to formation of the lipid-binding site [146,151,156]. The determined equilibrium dissociation constant for cholesterol binding (~3 mol%) is on the low end of the physiological cholesterol concentration in mammalian cells [50,151]. The level of cholesterol in membranes of animal cells can make up to 50 mol% of the membrane lipid content but varies between membrane systems and tissues. The ER and nuclear membrane usually contain 1-10 mol% cholesterol, which increases to about 10-25 mol% in Golgi stacks and 30–40% in the plasma membrane [50,162–169]. Thus, cholesterol is most likely tightly bound to C99 under physiological conditions. But where and how does cholesterol bind to the short C99 peptide? Previous studies suggested a possible lipid-binding site around the N-helix/N-loop/TM domain element [145,170]. Due to the *trans* ring junctions, cholesterol is a flat molecule (Fig. 3), and binding of the rigid cholesterol to the TM helix is generally expected to be entropically favored over an association of the helix with more flexible diacyl phospholipids. One face of

cholesterol, the α-face, is smooth. The opposed β-face carries two methyl groups (C18, C19), resulting in a rough side. Interestingly, residues Gly^{700}, Gly^{704} and Gly^{708} of the TM domain, which have been shown to play a crucial role in C99 homooligomerization, appear to be involved in formation of the cholesterol-binding site (Table 1 and Fig. 6), suggesting a competition of homodimerization and cholesterol binding at the glycine zipper motif [151]. The tandem GxxxG motif (+GxxxA) on the C99 TM helix provides a large flat surface spanning over about three helix turns near the extracellular space, which is well-suited to interact with the cholesterol molecule, allowing Van der Waals interactions between the lipid ring system and the TM helix. Cholesterol binds with its relatively rough β-face to the TM domain, whereas the smooth α-face is oriented towards the surrounding lipids. The two methyl groups of the β-face might intrude in protein cavities, resulting in tighter binding [162]. Binding of cholesterol might be further enhanced by hydrogen bond formation between Glu^{693} and Asn^{698} of the N-loop and the N-helix, respectively, with the 3β-OH-group of cholesterol. To optimize the interaction of cholesterol with the soluble regions of C99, a certain flexibility of the N-Loop is given, which allows the N-helix to rotate and to adapt a suitable position to interact with the cholesterol molecule in an induced-fit clamp-like manner [146]. Noteworthy, residues involved in cholesterol binding to C99 are different from postulated cholesterol-binding motifs found in other proteins (CRAC, CARC, CCM) [75,162]. Together, these results indicate that cholesterol binding of C99 depends on (i) the tandem GxxxG motif near the membrane surface, providing a flat attachment surface, (ii) a flexible loop at the membrane water interface and (iii) hydrogen bond formation between amino acids in the soluble region of the MP and the 3β-OH-group of cholesterol. In the presence of cholesterol, C99 preferentially interacts with cholesterol rather than with a second monomer to form a homodimer [151]. As the dimerization propensity of C99 is supposed to be low under physiological conditions, and as the interfaces for cholesterol binding and homodimerization highly overlap, cholesterol binding directly competes with TM helix dimerization [151].

However, does cholesterol binding to C99 have an effect on the proteolytic cleavage by the γ-secretase? Numerous reports associate β- and γ-secretase with cholesterol-rich lipid phases in membranes, where the amyloidogenic pathway is active [171–178]. The α-secretase, which is responsible for the non-amyloidogenic pathway, resides in the bulk membrane phase, indicating a specific cholesterol dependence of the amyloidogenic pathway [68,179]. Proteolytic cleavage of C99 by the γ-secretase is dramatically enhanced in cholesterol-containing membranes [150]. Whether cholesterol is bound to C99 during the cleavage process remains an open question. This may be the case, since C99 is cleaved directly below the residues involved in cholesterol binding. Therefore, cholesterol binding might preserve the C99 structure necessary for cleavage by the γ-secretase or acts as a molecular "glue" between C99 and the γ-secretase. The γ-secretase appears to cleave both dimeric and monomeric C99 substrates, while the cleavage of monomeric proteins alters the distribution of different amyloid β peptides that are generated [110,158,180,181]. While dimerization of C99 does not conflict with the observations made and also does not affect γ-secretase cleavage [180,181], cholesterol binding to C99 might directly activate γ-secretase cleavage [150] and at the same time directly inhibits α-secretase cleavage [182].

3. Summary: How could lipids control oligomerization of TM helices?

Tight binding of specific lipids to large, polytopic MPs is well described and it is clear that such lipids are intrinsic and essential parts of the MP structure, crucial for the stability and activity of the MP. Effects of global lipid properties, such as the hydrophobic thickness, the lipid head group chemistry, lipid asymmetry or the lateral pressure profile on the interaction of simple, single-span TM proteins have also been analyzed to some extent in the past decade. In contrast, binding of defined lipid species to individual TM helices has moved into the research focus only recently, and regulation of TM helix monomer–oligomer equilibria by binding of defined lipids is a concept, which has emerged only lately.

Lipids bind to single-span MPs, both in the juxta-membrane region as well as in the hydrophobic membrane core. The lipid head groups typically bind *via* electrostatic interactions and hydrogen bond formation at the membrane surface to the protein. Especially negatively charged lipid head groups appear to be recognized and bound by the extra-membrane regions following individual TM helices. However, in several cases – potentially in all cases – also the acyl chains of a given lipid bind to cavities at the surface of a TM helix *via* Van der Waals interactions. On the surface of the p24 and the C99 protein, regions have been identified, which allow defined packing of the hydrophobic lipid moieties. Nevertheless, an acyl chain region can hardly determine specificity in binding of a diacyl membrane lipid. Only the acyl chain length and/or the degree of saturation are possible determinants for specificity. Thus, it appears to be likely that many different lipids with identical acyl chains can transiently bind to a given TM helix but only lipids with both the correct acyl chain and head group bind more tightly and reside at a TM helix surface for a longer time span. In the case of cholesterol, binding of the hydrophobic region might already be highly specific and defined interactions in the hydrophilic region potentially stabilize the bound lipid.

As summarized here, lipid binding to single-span TM helices can affect the equilibrium between TM helix monomers and higher ordered oligomers. Some interactions counteract oligomerization and in other cases, lipid binding appears to enhance oligomerization. As reversible oligomerization is involved in activation of many MPs, binding of defined lipids to single-span TM proteins might be a mechanism to regulate and/or fine-tune the protein activity. But how could lipid binding trigger the activity of a protein? How can binding of a single lipid molecule to a TM helix affect the structure of a TM helix oligomer, and consequently its signaling state?

The thus far analyzed and here discussed examples of lipid-binding single-span TM proteins highlight some common grounds to be considered in further studies as well as in the design of novel lipid-binding TM sequences (Fig. 7).

1. It still is completely unclear, at which stage TM helix oligomerization and lipid binding eventually compete. Lipids could either bind to a preformed oligomer and thereby stabilize or weaken a TM helix–helix interaction. Alternatively, lipids bind to monomeric proteins and thereby either stabilize the monomeric state or generate an oligomerization-competent monomer. Cholesterol more likely binds to the monomeric C99 protein and thereby hinders formation of TM helix–helix contacts.
2. Binding of negatively charged lipid head groups to stretches of basic amino acids could simply shield clusters of positive charges, decreasing repulsion of positively charged protein regions. Thereby, the positive free energy term caused by the repulsion is diminished, resulting in increased stability of a TM helix oligomer.
3. If binding of a lipid head group is mediated by amino acids located on two different proteins, *i.e.,* if the lipid-binding domain is formed by two different proteins, binding of a lipid would result in formation of a dimeric structure. *E.g.*, in the case of the bacterial light-harvesting proteins, where two individual TM helices interact with pigments, individual pigments intercalate between the two individual TM helices and thereby stabilize the dimeric structure [183,184]. Similarly, a bound lipid could act as molecular "glue". This might occur in the soluble domains, but promoting and stabilizing TM helix–helix interactions in the hydrophobic TM region are also possible. Intercalation of individual lipids between different proteins is well described in the case of large, polytopic TM protein complexes [67].
4. As shown in the case of the Kir2 K^+ channels, binding of a lipid head

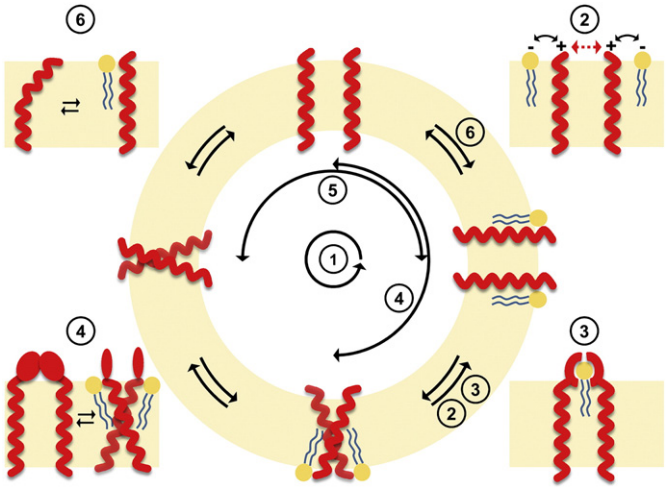

Fig. 7. Lipid binding influences TM helix oligomerization. The monomer–oligomer equilibrium of TM helices might be affected by lipid binding in different ways. (1) Lipids could bind to the monomeric or the oligomeric MPs, and lipid binding could promote or prevent oligomerization. (2) Lipid binding to positively charged amino acids could shield clusters of positive charges and could be involved in the proper positioning of a TM helix within the membrane plane. (3) A lipid-binding cavity might be formed in between two separate proteins, and thus the lipid could act as a molecular "glue". (4) Interaction of protein domains with a lipid can induce structural re-arrangements, preventing or promoting TM helix–helix interactions. (5) When a lipid-binding site overlaps with a dimerization motif, helix–helix interactions directly compete with helix–lipid interactions. (6) Lipid binding to a TM helix can influence the structural dynamics of a TM helix, which impacts TM helix–helix interactions. For further details see the text. The numbers refer to the respective categorization discussed in Section 3.

group to an extra-membrane domain of a protein can induce a conformational change in the extra-membranous regions, which results in reorganization of soluble domain interactions, altered steric constraints and finally in altered TM helix–helix interactions. This can then lead to the formation of a TM helix oligomer with an altered structure, favor formation of a TM helix oligomer or drive monomerization of an oligomer.

5. If a lipid binds in a TM region, which is also involved in TM helix oligomerization, as *e.g.,* observed in the case of the C99 TM helix dimer, lipid binding directly competes with TM helix oligomerization and thus, the local concentration of a lipid triggers the oligomeric state.
6. Lipid binding to a surface of a TM helix, which is not involved in formation of TM helix–helix contacts, might induce structural alterations in the TM helix, resulting in increased or diminished interaction propensities. Lipid binding could alter the helix flexibility, which might be required for stable TM helix–helix contacts. Alternatively, lipid binding could affect bending of a helix, which eventually also affects interaction propensities.

Transparency document

The Transparency document associated with this article can be found in the online version.

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