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Role of GxxxG Motifs in Transmembrane Domain Interactions

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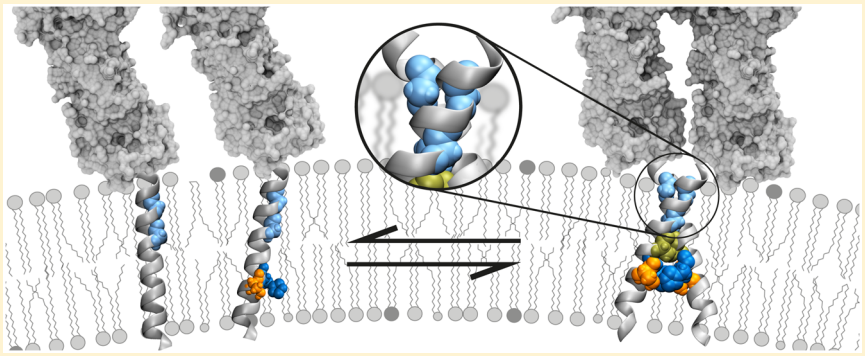

ABSTRACT: Transmembrane (TM) helices of integral membrane proteins can facilitate strong and specific noncovalent protein−protein interactions. Mutagenesis and structural analyses have revealed numerous examples in which the interaction between TM helices of single-pass membrane proteins is dependent on a GxxxG or (small)xxx(small) motif. It is therefore tempting to use the presence of these simple motifs as an indicator of TM helix interactions. In this Current Topic review, we point out that these motifs are quite common, with more than 50% of single-pass TM domains containing a (small)xxx(small) motif. However, the actual interaction strength of motif-containing helices depends strongly on sequence context and membrane properties. In addition, recent studies have revealed several GxxxG-containing TM domains that interact via alternative interfaces involving hydrophobic, polar, aromatic, or even ionizable residues that do not form recognizable motifs. In multipass membrane proteins, GxxxG motifs can be important for protein folding, and not just oligomerization. Our current knowledge thus suggests that the presence of a GxxxG motif alone is a weak predictor of protein dimerization in the membrane.

Integral membrane proteins account for ∼25% of all proteins and can be subdivided into single-pass (bitopic) and multipass (polytopic) proteins. Most membrane proteins pass the lipid bilayer by way of α-helical transmembrane domains (TMDs). β-Barrel membrane proteins are found in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts. It has been several decades since research on human glycophorin A (GpA) demonstrated clearly that TMDs could facilitate strong, specific protein−protein interactions (PPIs) in the membrane environment.[1,2] TMD−TMD interactions are vital to a number of cellular processes, and their inhibition may contribute to disease therapy.[3,4]

To which types of noncovalent structures do TMDs assemble? To date, only approximately two dozen high-resolution structures have been published that reveal distinct TMD helix−helix interfaces from single-pass proteins in structural detail (reviewed in ref 5, http://www.drorlist.com/nmr/MPNMR.html). Most of these structures are homo- or heterodimers, but some describe trimers,[6] tetramers,[7,8] and pentamers.[9] In some cases, homo- and heterodimerization are mutually exclusive as they utilize the same interface residues, as in the interaction of HLA II α−β subunits, and in integrin αIIb−β3 subunits.[10−14] For other proteins, the homotypic and heterotypic interactions utilize different interfaces, as proposed for the Na,K-ATPase β-subunit TMD.[15] For DAP12[16,17] and FtsB,[18,19] homodimer formation is thought to precede interaction with other TM helices, leading to complexes involving at least three protein subunits. TMD−TMD interactions can also support the noncovalent assembly of large membrane protein complexes[20] that may contain both single-pass and multipass subunits, as exemplified by the ATP synthase complex[21] or photosystem II.[22]

How extensive is the transmembrane domain (TMD) interactome? High-throughput methods such as the membrane yeast two-hybrid assay[23] or tandem affinity chromatography with mass spectrometry[24] can identify a large number of putative interactions between membrane proteins but do not distinguish between interfaces in the soluble or membrane-spanning domain. Several genetic reporter assays measure interactions directly between TMDs within the inner membrane of *Escherichia coli*, including ToxR, TOXCAT, GALLEX, BACTH (reviewed in ref 25), and also AraTM.[26] Systematic studies have used these assays to measure homotypic or heterotypic interactions among TMDs of 19 human receptor tyrosine phosphatases,[27] all 58 human receptor

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Table 1. Proteins That Show GxxxG-Dependent or GxxxG-Independent TMD Interactions

UniProt Accession	Protein name[a]	Topology	Interaction type[b]	Sequence[c]	Ref
GxxxG-dependent TMD interaction					
Q9UNQ0	ABCG2 TM1	multi-pass	homo	IAQIIVTVVLGLVIGAIYFGL	*118*
Q8NI60	ADCK3	single-pass	homo	LANFGGLAVGLGFGALA	*47*
Q8KRV3	ATPase c, TM1	multi-pass	homo & hetero	VVLAASAVGAGTAMIAGIGPGVGQG	*109*
Q12983	BNIP3	single-pass	homo	VFLPSLLLSHLLAIGLGIYIGR	*64,73,146,147*
P13688	CEACAM1	single-pass	homo	AIAGIVIGVVALVALIAVALACFL	*148,149*
Q12913	DEP1, RPTP	single-pass	homo	VICGAVFGCIFGALVIVTVGGFIFW	*27*
Q15303	ErbB4	single-pass	homo	LIAAGVIGGLFILVIVGLTFAVYV	*65*
P02724	GpA	single-pass	homo	ITLIIFGVMAGVIGTILLISYGI	*2,33*
P01909	HLA-DQα1	single-pass	homo & hetero	TVVCALGLSVGLVGIVVGTVFII	*10,11,32*
Q30167	HLA-DRβ1	single-pass	homo & hetero	MLSGVGGFVLGLLFLGAGLFIYF	*10,11*
P08514	Integrin αIIb	single-pass	homo & hetero	AIPIWWVLVGVLGGLLLLTILVLAMW	*14,68,150,151*
P20826	Kit-Ligand	single-pass	homo	WTAMALPALISLVIGFAFGALYW	*131*
P03107	L2	viral capsid	homo	ILQYGSMGVFFGGLGIGTGSGTG	*152*
P25189	Myelin protein P0	single-pass	homo	YGVVLGAVIGGVLGVVLLLLLLFYVV	*49*
O14786	Neuropilin 1	single-pass	homo & hetero	ILITIIAMSALGVLLGAVCGVVL	*30,153,154*
Q67482	prM TM1	multi-pass	homo & hetero	GYAFLAAALGWMLGSNNG	*155*
Q7L4S7	ARMCX6	single-pass	homo	VGWMAAGLMIGAGACYCVYKL	*32*
Q60492	Sigma1R, TM2	multi-pass	homo	WVFVNAGGWMGAMCLLHASLS	*119,120*
P34741	Syndecan 2	single-pass	homo	VLAAVIAGGVIGFLFAIFLILLLVY	*156*
O75056	Syndecan 3	single-pass	homo	AVIVGGVVGALFAAFLVTLLI	*156*
P31431	Syndecan 4	single-pass	homo	VLAALIVGGIVGILFAVFLILLLMY	*156*
P21731	TXA2 TM5	multi-pass	homo	GLLFSMLGGLSVGLSFLLNTV	*117*
Q48247	VacA	secreted domain	homo	VIIPAIVGGIATGTAVGTVSGLL	*94*
P03522	VSV-G	single-pass	homo	FFFIIGLIIGLFLVLRVGIHL	*43*
GxxxG-dependent interaction for one of multiple interfaces					
P05067	APP	single-pass	homo	GAIIGLMVGGVVIATVIVITLVML, GAIIGLMVGGVVIATVIVITLVML, GAIIGLMVGGVVIATVIVITLVML	*51,69,157,158*
P21709	EphA1	single-pass	homo	TGGEIVAVIFGLLLGAALLLGILVF, TGGEIVAVIFGLLLGAALLLGILVF	*142,159*
P29317	EphA2	single-pass	homo	IGGVAVGVVLLLVLAGVGFFI, IGGVAVGVVLLLVLAGVGFFI	*160,161*
P05026	Na/K ATPase β1	single-pass	homo & hetero	ILLFYVIFYGCLAGIFIGTIQVMLL, ILLFYVIFYGCLAGIFIGTIQVMLL	*15*
GxxxG-independent TMD interaction					
P81449	Yeast ATPase e	single-pass	hetero	NVLRYSALGLGLFFGFRNDMA	*110-112 113*
O43914	DAP12	single-pass	homo	GVLAGIVMGDLVLTVLIALAV	*16,17*
Q8IZU9	KIRREL3	single-pass	homo	VIIGVAVGAGVAFLVLMATIV	*32*

[a]Abbreviated names are shown. Information regarding full protein names, organism, and predicted transmembrane regions of each protein is available online from the UniProtKB database (www.uniprot.org).[145] [b]Homotypic interaction or heterotypic interaction. Heterotypic interaction partners are described in the relevant references. [c]Key residues thought to be involved in TMD−TMD interactions are underlined. In some cases, multiple interfaces have been predicted.

tyrosine kinases (RTKs),[28] 10 toll-like receptors,[29] 13 proteins associated with RTK or neuropilin signaling,[30] 15 human proteins selected on the basis of amino acid conservation patterns,[31] and 33 human proteins representing homology-based clusters.[32] Typically, the TMDs tested show a broad range of affinities; approximately 10−20% of a given sample self-interacts as efficiently as the strongly dimerizing human glycophorin A (GpA).[33−35] To understand the extent of the TMD interactome, there is a clear need for more large-scale studies that systematically test both homo- and heterodimerization of natural TMDs.

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GXXXG MOTIF

Discovery of GxxxG and Related Motifs. The GxxxG motif (x represents any amino acid) discovered in human GpA has now been implicated in TMD interactions for more then 20 different proteins (Table 1). Initially, Furthmayr and Marchesi[1] in 1976 found that the GpA transmembrane domain was responsible for the dimerization. Lemmon et al.[2] combined scanning mutagenesis with SDS−PAGE to identify LIxxGVxxGVxxT as the key motif in GpA homodimerization, a finding that was later confirmed by NMR spectroscopy.[36] The insertion of these residues into other hydrophobic sequence backgrounds also led to dimerization, leading to the concept of a "dimerization motif".[37,38] Selection of self-interacting TM sequences from random libraries yielded an abundance of GxxxG motifs,[39] demonstrating the importance of the motif for strong TMD−TMD interactions in vivo. Senes et al.[40] found that the GxxxG motif (also termed GG4) is highly over-represented in natural TMD sequences, suggesting that it has evolved as a key dimerization motif. Since these key findings, the GxxxG motif has been under intense study, and a number of GxxxG-mediated TMD interactions have been proposed for both single-pass and multipass α-helical membrane proteins (Table 1).

Selection of high-affinity TMDs from random libraries has also identified sequences with GxxxG-like motifs, also known as (small)xxx(small) motifs (Figure 1).[34,41−45,162] Small residues are typically defined as Gly, Ala, and Ser (Cys may be less crucial because of its low abundance in natural TMDs). The glycine zipper, where (small)xxx(small) motifs are concatenated to GxxxGxxxG and variants thereof, is also overrepresented in TMDs,[46] especially if they exhibit one-sided conservation, which suggests their interaction.[31] Glycine zippers are indeed associated with TMD interactions in a number of proteins.[15,47−51] Further examples of TMDs that interact via (small)xxx(small) motifs are found in a number of excellent reviews.[4,52−54] Helix−helix interactions based on (small)xxx(small) motifs are also found within multipass membrane proteins,[55] and soluble proteins[56] where they are thought to contribute to folding.

Structural Basis of Helix−Helix Interactions via (Small)xxx(Small) Motifs. Structural studies suggest that (small)xxx(small) motifs such as GxxxG can maximize interfacial van der Waals interactions and/or hydrogen bonding by allowing the interacting helices to be in the proximity of each other.[36,57,58] Thereby, the small residues facilitate interactions between the main-chain and hydrogen-bonding donors such as Ser and Cys.[59] In addition, direct Gly-Gly (main-chain/main-chain) interactions are thought to contribute to TMD affinity by CαH···O=C hydrogen bonding.[57,60−62] This is supported by recent studies in which the geometric requirements of these bonds have been determined and used to predict helix dimer structures.[47,63] Interactions via (small)xxx(small) motifs are often associated with the formation of right-handed dimers characterized by negative helix−helix crossing angles as in GpA (−40°),[36] BNIP3 (−34°),[64] ErbB4 (−40°),[65] and ErbB1 (−44°).[66] Walters and DeGrado[67] clustered helix pairs available from membrane protein crystal structures. The "GpA-like" parallel, right-handed cluster was termed GAS_{right} because of a distinct pattern of small residues and showed an average helix−helix crossing angle of −37.9°.[67] GxxxG motifs may also be important for interaction even if they are present on only one of two interacting helices as in the integrin αIIb

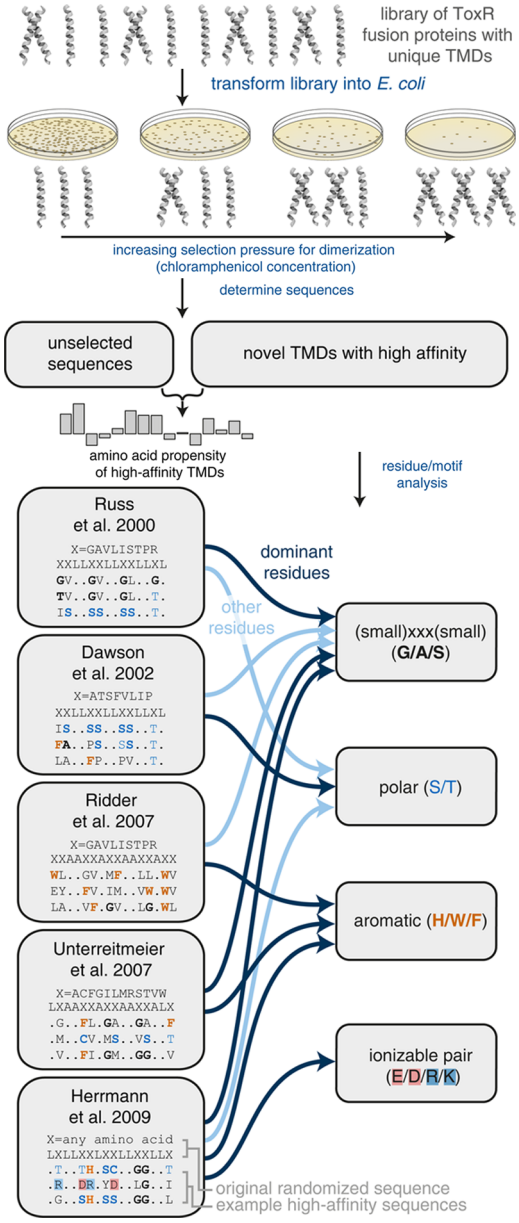

Figure 1. Artificial selection of strongly interacting TM helices using ToxR or TOXCAT methods in the *E. coli* cytoplasmic membrane. The method involves the creation of a library of plasmids, each encoding a ToxR fusion protein containing a partially randomized TMD. After transformation into *E. coli*, dimerizing TMDs convey antibiotic (chloramphenicol) resistance by promoting dimerization of the ToxR domain, which in turn upregulates a ToxR-dependent promoter and increases the level of expression of the antibiotic resistance gene. DNA sequencing of the plasmids within highly resistant *E. coli* leads to the discovery of sequences that promote TMD self-interaction (usually assumed to be dimerization). Each selection study utilized degenerate codons to allow a particular set of amino acids at each randomized position (X). For each study, the initial TMD sequence showing randomized (X) and fixed (Leu or Ala) positions is shown, and also three example "high-affinity" TMDs sequenced in the course of that study. At the lower right, a list of motifs and residues that were identified among the sequenced "high-affinity" TMDs is shown. The four residue types (small, polar, aromatic, and ionizable) have been emphasized in the studies mentioned above;[34,39,42−45] however, this is not a complete list, and many of the artificially selected sequences could be otherwise classified.

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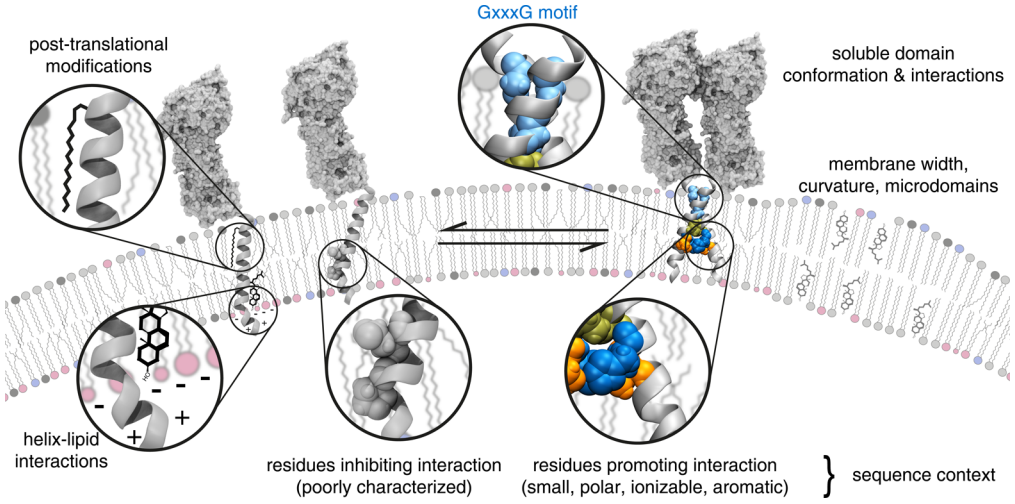

Figure 2. Factors affecting the affinity of TM helices that contain putative "dimerization motifs" such as GxxxG. A generic protein with a transmembrane dimer is used for illustrative purposes only, based on a chimera between the structures of the BNIP3 TM homodimer[142] and the QSOX soluble domain.[143]

and β3 heterodimer.[68] Note that these structural analyses also associate small interfacial residues, including (small)xxx(small) motifs, with left-handed helix pairs.[59,67] One NMR structure of the amyloid precursor protein TMD indeed revealed a left-handed, parallel helix pair that relies on a (small)xxx(small) motif.[69] In this case, the GxxxA motif facilitates the close approach of the helices, but bulky residues provide most of the van der Waals contacts.

Dependence of GxxxG Motifs on Sequence Context. The affinity of GxxxG-containing TM helices is strongly affected by sequence context, as illustrated in Figure 2 and discussed extensively in the literature.[5,70−72] Interfacial residues are typically identified by the impact of mutating them on the TMD interaction, their "mutation sensitivity". Ideally, this is done by mutating each wild-type residue to multiple amino acids, as conducted for GpA,[2] integrin αIIb,[14] BNIP3,[73] FtsB,[19] and ADCK3.[47] In the case of GpA, mutation-sensitive residues surrounding the GxxxG motif include L75 and T87, one type of sequence context within which GxxxG can form an efficient interface.[2,35,74] Also, it has been proposed that neighboring β-branched residues promote GxxxG-mediated dimerization by presenting a "preformed interface" that negates the loss of side-chain entropy associated with dimerization.[39,40] Neighboring residues such as leucine that have a high number of possible rotamers in the monomer would therefore convey an unfavorable decrease in entropy upon dimerization. Evidence of this hypothesis comes from the observation that β-branched residues frequently neighbor the Gly residues of high-self-affinity TMDs that were selected from randomized sequences.[39] However, our list of natural TMDs where GxxxG participates in the interface includes many representatives that lack β-branched residues (Table 1). The alignment of these sequences based on the GxxxG motif also shows no particular pattern or abundance of β-branched residues. More recent selection studies have not found an enrichment of β-branched residues near GxxxG (Figure 1).[34,43,44] Unfortunately, a detailed analysis of the amino acid enrichment in dimerizing TMD sequences is not possible, because most selection studies did not quantify the amino acid composition of unselected TMDs. To improve our understanding of the amino acid composition surrounding dimerizing GxxxG motifs, further selection studies are required.

Apart from scanning mutagenesis, the dependence of GxxxG-mediated dimerization on sequence context has been detected by the additive effect of double mutations[75] or by the requirement for non-GxxxG residues when the motif is reconstructed in a neutral sequence background.[35,37,76] In the cell, the influence of sequence context presumably ensures that the many GxxxG-containing TM helices do not undergo nonspecific interaction. Noninterfacial residues can also affect interaction by altering helix properties that indirectly affect TMD association. These properties may include the depth of membrane insertion, tilt angle, kinks/curves, backbone dynamics, or the location in microdomains (Figure 2). As described in detail elsewhere,[5,77−81] membrane properties, including the presence of specific lipids, curvature, lateral pressure, and surface charge, can all strongly affect TMD interactions.

PREDICTING HELIX−HELIX INTERFACES BASED ON GXXXG MOTIFS

GxxxG Motifs Are Abundant in Transmembrane Helices. A simple sequence-based attempt to predict TMD−TMD interfaces involves searching for characteristic amino acid motifs such as GxxxG. However, GxxxG motifs are relatively common because they are present in ∼12% of TM helices.[40,43] We show here that this proportion varies greatly between species, depending on the glycine content of their TMDs (Figure 3). This observation is also poorly compatible with the idea of GxxxG being a bona fide "dimerization motif", assuming that the organisms show a similar propensity for PPI in the membrane. Human TMD sequences are relatively glycine-rich (Figure 3). Single-pass TMDs containing at least one GxxxG motif are therefore almost twice as abundant in humans (15.6%) as in the yeast *Saccharomyces cerevisiae* (7.9%) or the nematode *Caenorhabditis elegans* (8.6%). It has been previously noted that GxxxG motifs in TMDs are ∼32% more abundant than expected on the basis of a random distribution of glycines.[40] The question of how many of these GxxxG motifs are involved in TMD interactions remains. At present, this can only be suggested because of the limited number of case studies

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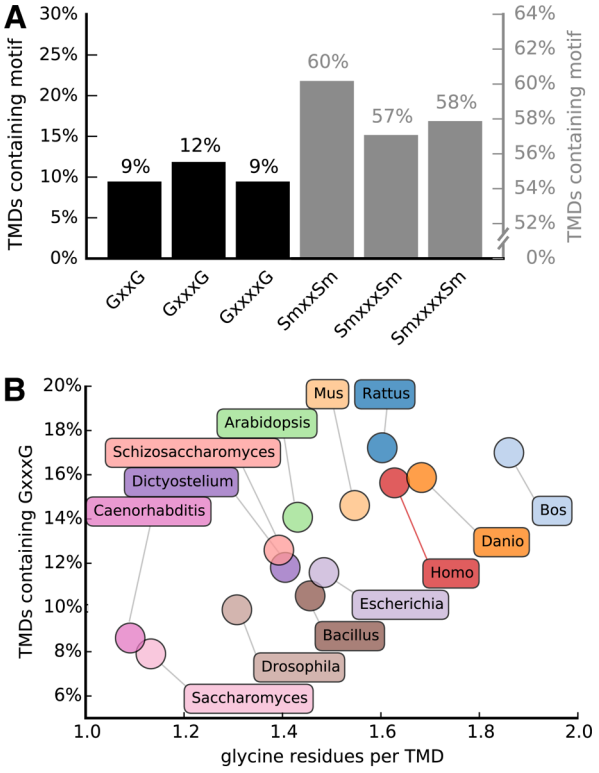

Figure 3. (Small)xxx(small) motifs are abundant in TM helices of single-pass membrane proteins. (A) Percentage of single-pass TM helices containing at least one motif. G = Gly; Sm = small (Gly, Ala, Ser, or Cys), and x = any separating residue. A nonredundant data set was obtained by using UniRef50[144] representatives. The residues corresponding to the transmembrane domain were derived from the UniProtKB annotation.[145] (B) Correlation between GxxxG abundance and glycine content within TMDs. The UniProtKB database of all single-pass proteins was filtered to contain only organisms with more than 80 proteins. A nonredundant data set was created for each organism by selecting only a single representative sequence from UniRef50 clusters. Organisms with fewer than 80 nonredundant sequences were again excluded, leaving 13 organisms with a median of 232 nonredundant protein sequences, and an average of 1.46 glycines per TMD. The organisms (*C. elegans*, *S. cerevisiae*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, *Bacillus subtilis*, *E. coli*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Danio rerio*, and *Bos taurus*) were assigned arbitrary colors. Linear regression yields an R^2 value of 0.82.

that have combined mutagenesis of GxxxG motifs with interaction assays. At least one (small)xxx(small) motif is present in 56% of single-pass TM helices (Figure 3). Again, it seems likely that only a proportion of these motifs are involved in TMD interactions.

Not All GxxxG Motifs Are Functionally Significant. There are cases in which the mutation of a GxxxG motif in the TMD does not disrupt known protein function. For example, the GxxxG motif originally proposed to drive oligomerization of the SARS Cov S protein[82] was later suggested to be dispensable for protein function.[83] The GxxxG motif within the major coat protein TMD of the Ff bacteriophage (M13) has been proposed to facilitate dimerization,[35] although that TMD is essentially monomeric in the TOXCAT assay.[35] The M13 GxxxG motif is not strictly required in the viral lifecycle, as polymorphisms to other small residues are found in viable mutants,[84] and polymorphisms to Ala or Val are found in homologs.[85] A recent high-resolution structure of the capsid does not suggest a key role for the GxxxG motif.[85]

GxxxG Motifs Do Not Always Convey Dimerization. The observations cited above already suggest that the presence of GxxxG or (small)xxx(small) motifs within interacting sequences is not a guarantee that they are involved in dimerization.[71,86] This is supported by systematic analyses. An extensive study of human single-pass TMDs found that the mutagenesis of (small)xxx(small) motifs disrupted self-interaction in some but not all cases.[32] There was also no clear link between the presence of a (small)xxx(small) motif and the level of self-affinity.[32] Several GxxxG motif-containing human single-pass proteins exhibit a low level of homodimerization in reporter assays, including the receptor tyrosine phosphatases PTPβ, PTPμ, and PTPρ,[27] the RTKs CCK4, INSRR, EphA1, and MET,[28] NETO2,[32] and the ubiquitin-conjugating enzyme E2 J2.[31] We do not exclude the possibility that some of these studies contain false negatives. For example, in ToxR-based assays, not all researchers vary the design of their reporter constructs to achieve the optimal orientation of a given helix–helix interface relative to the transcription activator domain.[32] However, screening of thousands of sequences using SDS–PAGE, in which the interaction of TMDs is not expected to depend on its orientation within the construct, has shown that the vast majority of (small)xxx(small) motifs do not convey dimerization.[87]

Even the presence of a GxxxG motif that is highly conserved among homologues is not a guarantee that the motif is involved in TMD interactions. Conserved TMD residues could be involved in a variety of functions, for example, enhancing helix flexibility,[88,89] lipid interactions,[78] cell localization,[90] or the correct orientation of soluble domains.[91] Interestingly, a broad motif analysis of transmembrane proteins includes a number of (small)xxx(small) motifs associated with cofactor binding.[92] As would be expected, most highly conserved GxxxG motifs have been shown to have an important functional role, but in several cases, a role in TMD interaction remains unclear. Examples include the GxxxG motif of *E. coli* DjlA[93] and the outer glycines of the tandem GxxxG motif of the secreted bacterial toxin VacA.[94] Recent research suggests that the entire GxxxG-containing hydrophobic domain of secreted VacA is not actually required for pore forming activity as originally proposed and instead has a role in mitochondrial targeting.[95]

Many Helix–Helix Interfaces Do Not Rely on (Small)xxx(Small) Motifs. The selection studies (Figure 1) and many case studies emphasize that TMD interactions can be driven by a diverse range of residue types, with broad tendencies toward small, polar, aromatic, and ionizable residues. In fact, for each of the 20 natural amino acids, we were able to find at least one example in the literature in which that residue is implicated in a given TMD–TMD interaction. In a design alternative to the right-handed GxxxG-containing TMD pairs, residues forming TMD–TMD interfaces frequently form a heptad sequence pattern (usually imperfect) that is associated with left-handed helix pairs due to the geometry of "knobs into holes" packing of side chains.[60,67,96] As in (small)xxx(small)-driven TMD interfaces, each interacting TM heptad pattern represents a unique combination of interfacial residues with interaction driven by van der Waals, hydrogen bonding, ionic, π–π, or cation–π interaction forces (as reviewed recently by Hong[61]).

A number of TMDs that contain GxxxG or (small)xxx(small) motifs have been shown to interact through an alternative, GxxxG-independent interface (Table 1). The GxxxG motif of

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ErbB2 is at the interface of neither the TMD homodimer nor the heterodimer with ErbB1, where instead TMD interactions are driven by a polar/small TxxxSxxxG motif.[97,98] The GxxxG motif of DAP12 has not yet been shown to play a role in TMD interactions, and instead, NMR[16] and TOXCAT studies[17] have revealed dimerization via polar residues D50 and T54.

In Multipass Membrane Proteins, GxxxG Motifs Can Facilitate Intramolecular Protein Folding. On the basis of the many studies showing the importance of small residues in the folding of multipass membrane proteins,[55,59,67,99] it is clear that GxxxG motifs within multipass proteins can be involved in both intermolecular (PPI) and intramolecular (folding) helix−helix interfaces. As yet, we are not aware of any study that has systematically analyzed whether GxxxG motifs have a higher propensity for intermolecular (PPI) interfaces than intramolecular (folding) interfaces do. A number of studies have investigated the role of GxxxG as a "dimerization motif" in multipass proteins where structural information is unavailable.[100−104] For the GxxxG motif in TM3 of hCTR1, a recent structural study suggests that the GxxxG motif is not directly involved in oligomerization and instead facilitates folding through contacts between TM1 and TM3.[105] Similarly, for both the GPCR secretin receptor[106] and the human proton-coupled folate transporter,[107] it has been proposed that GxxxG motifs mediate intramolecular packing, rather than a direct role in PPI. Other studies show the functional importance of GxxxG motifs in protein complexes without speculating about whether the motifs are involved in intermolecular or intramolecular interactions, such as an excellent recent analysis of the mitochondrial Tim23 complex.[108] The demonstrated role of GxxxG in the TM1−TM1 and TM1−TM2 interactions of the bacterial ATP synthase subunit c is another example of the potential importance of this motif in inter- and intramolecular TMD interactions.[109] In ATPase of yeast, rather than bacteria, the presence of GxxxG motifs has provided little information about potential TM helix−helix interaction partners, however. The GxxxG motif of subunit e is required for the recruitment of subunit g to the complex and is implicated in the supramolecular dimers and oligomers formed by ATPase complexes.[110−112] Although subunit e was expected to dimerize,[110] a later NMR study revealed the TMD in DPC micelles to be monomeric.[113] Some of the supramolecular interactions appear to be independent of the GxxxG motif-containing e/g subunits,[114,115] and most of the relevant TMD interfaces remain unclear.[21]

In eukaryotic cell assays, the effects of mutations can be misinterpreted because of the complexity of membrane protein biosynthesis. Indeed, some mutations within a TM helix of a multipass protein have disrupted not only oligomerization but also the amount of protein correctly inserted into the membrane and transported from the endoplasmic reticulum to the plasma membrane.[102,104,116] This could be interpreted as a sign that oligomerization is necessary for protein localization.[116] Indeed, cells retain some proteins in the endoplasmic reticulum until they are correctly assembled,[90] but how can mutations that affect oligomerization be distinguished from those that merely lead to misfolding? Strong evidence of the direct role of particular residues in PPI comes from studies in which a mutation has affected oligomerization but not surface expression. An example is the GxxxGxxxL triple mutant within TM5 of the TXA_2 receptor.[117]

To obtain more direct evidence that mutations affect oligomerization and not protein folding, some studies of multipass proteins have measured homodimerization of TM helices expressed individually as single-pass proteins in the ToxR system.[104,118−121] These techniques raise a number of technical questions: Is the length and hydrophobicity of an isolated TM helix from a multipass protein sufficient to allow correct membrane insertion?[53] Does expression as a single-pass protein affect TM helix tilt and structure? Therefore, what is the likelihood that interactions between isolated TM helices faithfully reflect assembly of multipass proteins? Proteins that contain only two or three TM helices seem less likely to be affected by these issues.[120,121] In contrast, TOXCAT of the six individual TM helices of ABCG2 did not appear to be useful in finding the dimerization interface.[118] Although GxxxG-dependent TM1−TM1 interactions were detected in the reporter assay, the modeling experiments conducted in the study suggest an interface via antiparallel helices TM1 and TM6.[118] These problems could be avoided by adapting single-pass bacterial reporter assays to full-length multipass proteins, as already demonstrated for ToxR,[122] or by utilizing the various membrane protein oligomerization assays established in yeast[23,104,123,124] or mammalian cells.[125]

Methods for Strengthening the Prediction of TMD−TMD Interaction. As outlined above and discussed in other reviews,[5,71] GxxxG and (small)xxx(small) motifs do not appear to be strong and decisive indicators of dimerizing TMDs; their presence is therefore only a weak predictor of an interaction. How could the predictive power of these motifs be improved? Arguably, motif searches can be replaced entirely with more sophisticated algorithms that predict residues likely to be in contact with other helices.[126] Other approaches could combine simple motif analyses with complementary prediction methods as outlined in the following. Helix−helix interfaces in membrane proteins are on average more conserved than helix−lipid interfaces,[127,128] a feature used for more than 10 years to improve structural predictions of multipass proteins.[129] Several case studies of single-pass TMDs also show a high degree of evolutionary conservation of interfacial residues, which supports their role in TMD−TMD interaction, including human HLA(MHC) II,[10] integrins,[130] Na,K ATPase,[15] the Kit ligand,[131] and *E. coli* FtsB.[19] The sidedness of conservation in TM helices of human single-pass proteins has also been associated with an increased level of homodimerization.[31] Another aspect of sequence conservation is the co-evolution of amino acids, which has been used to detect interacting helices within multipass proteins,[132−137] to identify PPI partners, including interacting membrane helices,[138] and also to predict dimer structures of TMDs from single-pass proteins.[139]

Force field-based modeling of TMD interactions can also be combined with motif and conservation analyses to improve the prediction of interfacial residues. Although such methods are computationally expensive, recently some simplified approaches have been developed that are compatible with the screening of a large number of TM helices. For example, the PREDDIMER algorithm[140,141] has been validated against several helix pairs with available NMR structures[139,141] and is available via a web server (http://model.nmr.ru/preddimer/). The "Cα Transmembrane" (CATM) method developed by the Senes lab initially screens pairs of TM helices for local geometry that allows CαH···O═C hydrogen bonding, using precalculated sequence-based rules.[63] This method has assisted in the rapid generation of dimer structures for several right-handed helix pairs.[47,63] The CATM program is available as an open-source C++ library (http://msl-libraries.org).

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CONCLUSIONS

Many TMD interactions require the close approach of the helices to maximize hydrogen bonding and/or van der Waals interactions and therefore depend on small residues at the helix−helix interface. Evolution has therefore repeatedly selected for TMD sequences that depend on a GxxxG motif for interaction and function. The recent literature contains many excellent case studies of GxxxG-mediated TMD interactions. However, in single-pass proteins, not all GxxxG motifs have roles in helix−helix interactions, and in multipass proteins, GxxxG motifs seem more likely to be involved in protein folding than in oligomerization. That (small)xxx(small) motifs frequently do not contribute to TMD−TMD interfaces can be explained by their known dependence on sequence context. For the prediction of TMD interactions, motif analyses can be strengthened by combination with other prediction techniques and should be complemented by experimental studies.

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ABBREVIATIONS

BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; GpA, glycophorin A; GPCR, G protein-coupled receptor; hCTR1, human copper transporter 1; HLA, human leukocyte antigen; MHC, major histocompatibility complex; NMR, nuclear magnetic resonance spectroscopy; PPI, protein−protein interaction; RTK, receptor tyrosine kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TM, transmembrane; TMD, transmembrane domain.

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