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Review

Transmembrane Domain Recognition during Membrane Protein Biogenesis and Quality Control

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One-fourth of eukaryotic genes code for integral membrane proteins, nearly all of which are inserted and assembled at the endoplasmic reticulum (ER). The defining feature of membrane proteins is one or more transmembrane domains (TMDs). During membrane protein biogenesis, TMDs are selectively recognized, shielded, and chaperoned into the lipid bilayer, where they often assemble with other TMDs. If maturation fails, exposed TMDs serve as a cue for engagement of degradation pathways. Thus, TMD-recognition factors in the cytosol and ER are essential for membrane protein biogenesis and quality control. Here, we discuss the growing assortment of cytosolic and membrane-embedded TMD-recognition factors, the pathways within which they operate, and mechanistic principles of recognition.

Introduction

Integral membrane proteins are an extremely diverse class of proteins that represent 20–30% of the protein-coding genes of all organisms [1]. They are essential for cellular function, with critical roles in signal transduction, organelle biogenesis, intracellular trafficking, small molecule transport, and cell adhesion. The endoplasmic reticulum (ER) is the site of initial assembly for nearly all membrane proteins in eukaryotes [2]. Structurally, the defining feature of an integral membrane protein is one or more transmembrane domains (TMDs), which are typically stretches of predominantly hydrophobic amino acids that span the lipid bilayer as alpha helices [3]. The integration of TMDs into the membrane, the decisive event in the biogenesis of all membrane proteins, poses two major biophysical challenges that must be overcome by the targeting and insertion machinery.

First, the protein synthesis machinery resides in the cytosol, so membrane proteins are initially made in an aqueous environment where they are intrinsically insoluble. This means that before a membrane protein can be stably inserted into the lipid bilayer, it must necessarily transit through an inhospitable medium. Thus, specialized factors are needed to recognize and shield TMDs in the cytosol until they can engage with the insertion machinery at the destination membrane [4,5]. Without such factors, TMDs are prone to potentially toxic aggregation and inappropriate interactions.

Second, TMDs vary widely in their sequence and biophysical properties [6], making their recognition in the cytosol and at the membrane challenging. TMDs are typically 12–35 residues long, share no unifying sequence features, and vary widely in hydrophobicity, helical propensity, and context within a protein. Hydrophobicity analysis shows appreciable overlap between bona fide TMDs and hydrophobic segments of soluble proteins [7]. Yet, the biosynthetic machinery in the cytosol and membrane must make this distinction with high fidelity. Ultimately, TMD recognition is the critical and decisive step made by the membrane-embedded machinery which partitions them from an aqueous to lipid environment [2,8].

These two central challenges, effective chaperoning and high-fidelity recognition, highlight the essential functions played by TMD-recognition factors throughout the biosynthetic process. Cytosolic factors all share the unifying function of shielding their TMD clients from solvent. In addition, these factors are often coupled, usually via protein–protein interactions, to either receptors at the ER for targeting, or ubiquitination machinery for degradation. TMD-recognition factors at the membrane typically provide a conduit between the cytosol and the interior of the lipid bilayer to facilitate either insertion or dislocation. These factors must display selectivity, with access between the cytosol and membrane usually being gated.

In this review, we discuss the functions and mechanisms of action of TMD-recognition factors. While some of these have a distinct role within a single dedicated pathway, others are increasingly appreciated to be ‘generalists’ that interface with multiple biological processes. For this reason, we have not organised the article around individual targeting, insertion, and quality control pathways. Instead, the article is segregated by factors that act in the cytosol and those that act in the membrane. In both sections, we seek to highlight shared principles of TMD handling between otherwise unrelated factors.

Cytosolic Factors for TMD Recognition and Shielding

Two conceptually different strategies have evolved to solve the problem of shielding a TMD between its initial synthesis and eventual insertion at the membrane. In the co-translational strategy, recognition and shielding are coupled temporally to protein synthesis and physically to the ribosome (Figure 1A). This strategy is defined by signal recognition particle (SRP), which is precisely positioned at the ribosomal exit tunnel to effectively eliminate exposure of the TMD to bulk cytosol (Figure 1B). In cases where SRP cannot or does not recognize TMDs, they are engaged post-translationally (Figure 1C) by a series of alternative factors. Because these recognition factors are not intimately coupled to the ribosome, post-translational handling of TMDs typically involves their transient exposure to the cytosol, a more dynamic mode of interaction, and comparatively abundant cytosolic factors. Each of the different cytosolic TMD-binding factors confers different degrees of commitment toward different eventual fates (Figure 1C), dictated primarily by the

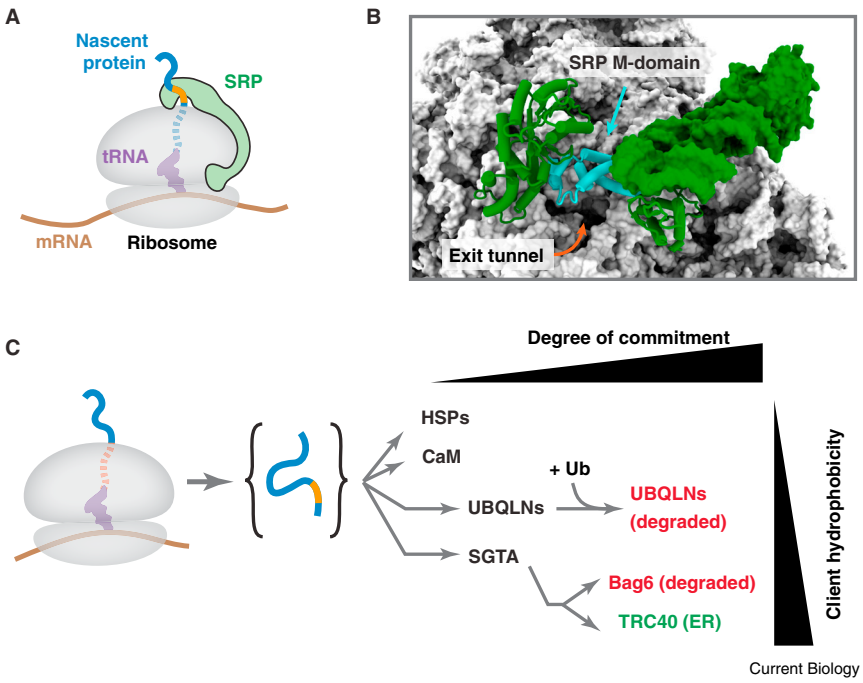

Figure 1. Co- and post-translational membrane protein recognition in the cytosol.
(A) Co-translational recognition of hydrophobic targeting elements (signal sequences and TMDs; orange) by signal recognition particle (SRP, green) at the ribosome. (B) Structure of the mammalian SRP–ribosome complex (PDB code 3JAN). SRP (green and cyan) is intimately associated with the ribosome (grey) and positioned precisely at the ribosomal exit tunnel. The M domain of the SRP54 subunit (cyan) recognizes signal sequences and TMDs. (C) Logic of post-translational TMD-binding proteins arranged approximately by their relative client hydrophobicity preferences (y-axis) and degree of commitment to a particular fate upon substrate binding (x-axis). Ubiquilin family members (UBQLNs) are non-committal until the bound substrate is conjugated with ubiquitin (Ub), resulting in commitment for degradation. HSPs, heat-shock proteins; CaM, calmodulin. Green indicates a biosynthetic fate, red indicates a degradative fate, and black indicates that the substrate's fate is un-committed.

rate of substrate dissociation and factor interactions with downstream partners.

Signal Recognition Particle

TMDs emerging from a translating ribosome in the cytosol are recognized by SRP [5]. In eukaryotes, SRP also recognizes cleavable amino-terminal signal sequences via their ~7–15 residue hydrophobic domain. The essential functional features of SRP are its ability to bind the ribosome at the mouth of the exit tunnel, its capacity to co-translationally recognize hydrophobic sequences, and its GTP-dependent interaction with the ER-localized SRP receptor [5,9,10]. These universally conserved activities permit TMD recognition, TMD shielding in the cytosol, and targeting of the ribosome–nascent-chain complex to the Sec translocation channel. This core framework for SRP function, developed in the early 1980s [11], remains surprisingly unchanged to the present. However, new technologies are now providing proteome-wide views of SRP's substrate range in vivo, and molecular views of its mechanism of signal recognition.

The earliest biochemical analyses established substrate hydrophobicity as a key parameter mediating SRP recognition and targeting [12,13]. This was reinforced by in vivo experiments showing that proteins whose biogenesis was impacted most by SRP depletion also contained the most hydrophobic targeting sequences [14–16]. Unaffected proteins were assumed to either not use SRP, or effectively use an alternative mechanism in its absence, a distinction that could not be easily resolved. Beyond verifying the importance of hydrophobicity and pointing to membrane proteins as a particularly sensitive class of clients, these studies left ambiguous the full complement of proteins that flux through SRP under normal conditions.

Recent studies have queried wild-type cells to identify the mRNA positions of all ribosomes affinity-purified via SRP [17,18] (Figure 2A). In *Escherichia coli,* SRP engages essentially all membrane proteins that co-translationally expose a TMD, as well as a subset of secretory proteins with particularly hydrophobic signal peptides [17]. This matches well with earlier work showing that the *E. coli* SRP is primarily needed for membrane proteins [15]. In *Saccharomyces cerevisiae*, the client range is broader: SRP engages the vast majority of ER-destined TMD- and signal-sequence-containing proteins [18], with no obvious correlation between engagement and target-sequence hydrophobicity (Figure 2B). The only types of ER-destined proteins not systematically recovered with SRP were secretory and membrane proteins whose sole hydrophobic domain is within ~60 residues of the carboxyl terminus. This class includes the tail-anchored membrane proteins [4], and very short secretory and membrane proteins [19,20]. The proximity of their hydrophobic domains to the stop codon means that SRP does not have enough time to effectively engage them during translation at the ribosome, explaining why they were not recovered with SRP.

Remarkably, a substrate's genetic requirement for SRP [16] was completely uncorrelated with whether SRP engages it under normal conditions (Figure 2C). Thus, although strong genetic dependence on SRP is a compelling (but not definitive) indicator of its physical engagement, SRP-independence cannot be equated with lack of SRP engagement. Presumably, SRP-independence instead reflects a protein's capacity to avoid aggregation or degradation before using another route to the membrane [16,21,22]. Consistent with these conclusions, acute SRP depletion in yeast showed de-localization from the ER of nearly all SRP-dependent mRNAs and around half of SRP-independent mRNAs [23]. Thus, with the exception of tail-anchored proteins, SRP physically engages — and is functionally necessary for — essentially all ER-destined membrane proteins. By contrast, a subset of ER-destined soluble proteins can be effectively imported into the yeast ER in the absence of SRP despite engaging and utilizing it under normal conditions.

Studies in yeast have searched for such an alternative route and recently identified three interacting proteins (termed SND1, SND2, and SND3, for 'SRP-independent') that are capable of

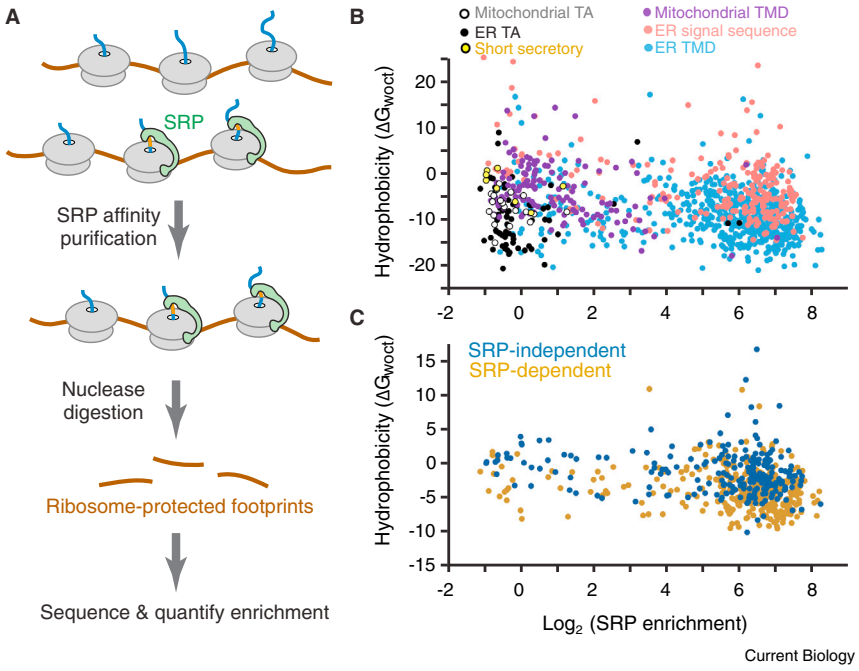

Figure 2. The client range of eukaryotic SRP.

(A) Method to identify SRP substrates via affinity purification of SRP-containing polysomes and deep sequencing of ribosome-protected mRNA fragments [18]. (B) SRP-affinity purification data of Chartron *et al.* [18] segregated by protein type and nature of the first hydrophobic element. The Log_2 enrichment of individual substrates by SRP affinity purification (x-axis) is plotted relative to the summed hydrophobicities (ΔG_{woct}) of all amino acids in the targeting element (y-axis) calculated by the method of White and Wimley [147]. On this scale, hydrophobic and hydrophilic residues have negative and positive ΔG_{woct} values, respectively. Short secretory proteins were defined as those containing a signal peptide and overall length of 100 residues or less. Tail-anchored (TA) proteins were defined as proteins with a single TMD within the last 60 residues of the protein. Annotations of protein localization were from Uniprot; those with uncertain or dual localization were excluded. Note the very clear segregation of ER-destined non-tail-anchored and non-short proteins (blue and pink) from all others. No systematic difference in SRP enrichment is seen between signal sequences and TMDs (TMD). (C) Relationship between SRP interaction [18] and dependence on SRP for translocation as determined by Ast *et al* [16]. Although there is a statistically significant difference in hydrophobicity between genetically defined, SRP-dependent (blue) and SRP-independent (gold) targeting sequences, no difference is observed in their relative enrichment by SRP-affinity purification.

partially suppressing loss of SRP. SND2 physically interacts with the Sec translocon, linking these proteins to some aspect of protein translocation. However, their mechanism of function remains to be elucidated. For the time being, available genome-wide studies indicate that SRP handles the vast majority of secretory- and membrane-protein targeting to the ER, and has broad specificity based on hydrophobicity of the targeting sequence. Different species may have different hydrophobicity thresholds for SRP binding, likely explaining why most signal peptides are recognized by SRP in eukaryotes [18], but not *E. coli* [17].

The molecular basis of SRP preference for hydrophobic sequences has come from structural analysis. In eukaryotes, SRP is a ribonucleoprotein complex composed of an approximately 300-nucleotide RNA scaffold and six protein subunits. The key functional component is SRP54 (Ffh in *E. coli*), whose methionine-rich M domain is responsible for engaging signal sequences and TMDs. When SRP is bound to the ribosome, the M domain is precisely positioned at the ribosomal exit tunnel [24,25], an ideal location to sample nascent chains as they emerge (Figure 1B). The crystal structure of the Ffh M domain revealed a hydrophobic groove formed by alpha-helices enriched with methionine residues [26]. Subsequent structures of the isolated M domain bound to a hydrophobic peptide showed that it resides within the hydrophobic groove [27–29]. The different structures have somewhat different arrangements of the M-domain alpha helices, suggesting a flexible scaffold that can conform to different substrates.

More recently, advances in cryo-electron microscopy have permitted the determination of increasingly higher resolution structures of native ribosome–nascent-chain complexes with SRP before and after substrate binding (Figure 3A). These structures not only allowed earlier high-resolution X-ray structures of isolated domains to be docked into the native complex, but also revealed a mechanism for how SRP might impose a hydrophobicity threshold for substrate binding [30]. In an unengaged state, the hydrophobic groove of the M domain was observed to be auto-inhibited by an amphipathic carboxy-terminal 'placeholder' helix (Figure 3A). Notably, the carboxy-terminal domain was omitted in all earlier X-ray structures, explaining why this was not observed previously. In the engaged structure, the carboxy-terminal helix is displaced by the substrate's TMD to a position where it serves as a 'lid' over the substrate-bound hydrophobic groove.

These observations suggest that the hydrophobicity threshold for SRP binding is effectively set by the biophysical properties of the auto-inhibitory helix: displacement of this helix can only occur when its hydrophobicity is exceeded by the substrate. The auto-inhibitory helix also prevents constitutive exposure of SRP's hydrophobic groove. This probably explains why free SRPs cannot effectively bind hydrophobic sequences promiscuously, unless they are presented in a constrained environment at high local concentrations at the ribosomal exit tunnel. However, these considerations still cannot easily explain why mitochondrial membrane proteins are avoided. Indeed, the hydrophobicity of the first TMD in mitochondrial membrane proteins is not appreciably different than ER signals and TMDs (for example, see Figure 2B), yet only the latter are engaged by SRP. The nascent polypeptide-associated complex is implicated in aiding this discrimination [31,32], possibly by modulating SRP–ribosome and ribosome–membrane interactions [33–35].

Both genome-wide studies and biochemical analysis suggest that SRP can be specifically recruited to the ribosome before the TMD emerges from the exit tunnel [30,36], or in some cases,

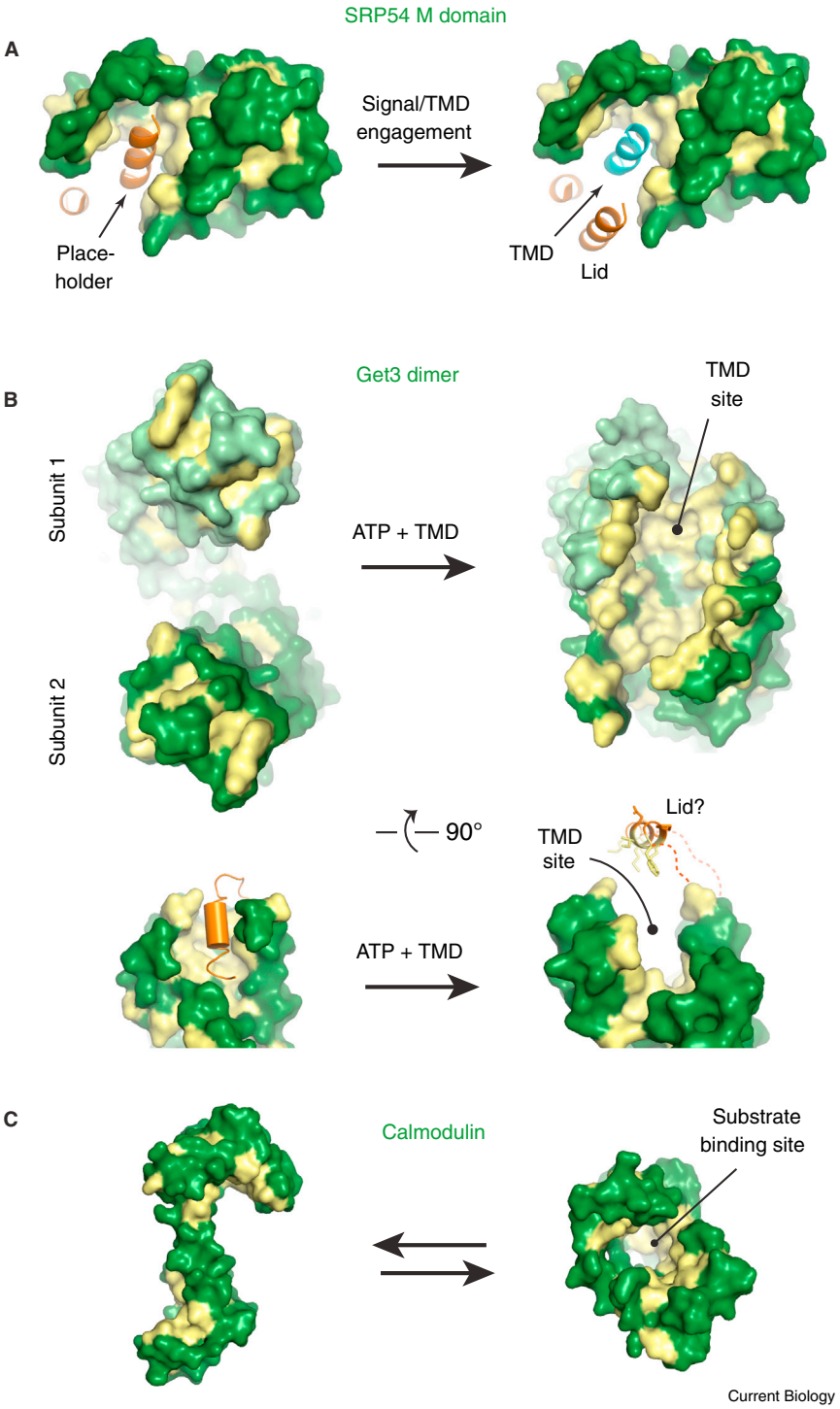

Figure 3. Structural basis of TMD binding by three different cytosolic factors.
(A) Structures of the M domain of mammalian SRP54 without (left) and with (right) a TMD substrate (cyan). Hydrophobic residues (Leu, Ile, Val, Met, Phe, and Trp) are shown in yellow. Two amphipathic carboxy-terminal alpha helices are shown in orange, one of which acts as a 'placeholder' in the substrate-binding groove of the substrate-free structure, and as a 'lid' in the substrate-bound structure. PDB codes: 3JAN, 3JAJ. (B) Structures of the yeast Get3 homodimer in the nucleotide-free apo state (left) and ADP•AlF_4^- state (right). The helical subdomains of the two subunits are separated in the apo structure. They do not expose large hydrophobic surfaces (in yellow) due to an amphipathic helix (orange cylinder) that occludes them. In the ADP•AlF_4^- structure, which is similar to the ATP-bound state, the helical subdomains re-arrange and come together to form a large composite hydrophobic groove shown to house the substrate TMD. The amphipathic helix is not ordered in this structure, but photo-cross-linking experiments to a bound substrate suggest it may form a lid to shield the substrate. More speculatively, the same amphipathic helix might be housed inside the hydrophobic groove as a placeholder until substrate binding. PDB codes: 2WOO, 2WOJ. (C) Structures of the open, substrate-free conformation and closed, peptide-bound conformation of mammalian calmodulin. The peptide substrate is not shown to visualize the hydrophobic substrate-binding site. PDB codes: 1CLL, 2VAY.

before the hydrophobic domain is even synthesized [18]. The benefits of pre-recruitment are obvious: it would allow SRP to capture hydrophobic sequences before any appreciable exposure to aqueous solvent and without any competition from more abundant chaperones or TMD-binding factors. It remains unclear how SRP can be recruited to some but not other ribosomes without relying on direct nascent-chain interaction. Because ribosome conformation during translation can vary in a codon-specific manner [37], it has been speculated that these differences might be exploited to recognize those ribosomes decoding strings of hydrophobic residues [30]. Alternatively, some distinguishing aspect of the pioneer round of translation has been speculated to facilitate SRP pre-recruitment [18]. In general, the relationship between SRP and translation remains to be clarified given that the original finding of SRP halting elongation in a heterologous plant–mammal hybrid in vitro system [38] was not recapitulated in subsequent biochemical and ribosome-profiling experiments in homologous systems [18,39–41].

TRC40/GET3 and Tail-anchored Protein Targeting

It has long been appreciated that some membrane proteins do not have access to the SRP-dependent pathway [42]. One such class is the tail-anchored proteins, defined by a single TMD close to the carboxyl terminus. The proximity of the TMD to the termination codon means that translation terminates before the TMD can exit the ribosomal tunnel (which can accommodate approximately 40 amino acids of a nascent polypeptide), thereby precluding co-translational SRP engagement. These proteins must instead rely on post-translational recognition and delivery to the ER. This parallel pathway was discovered about 10 years

ago [43–45] and is now understood in substantial mechanistic detail in yeast and higher eukaryotes [46,47].

The central TMD-recognition factor in this pathway is TRC40 (Get3 in yeast), a widely conserved ATPase that mediates tail-anchored protein targeting to the ER [43]. Crystal structures show that Get3 is a symmetric homodimer, with each monomer comprised of an ATPase domain and an α-helical domain [48–52]. The conformation of the α-helical domain is sensitive to the nucleotide state of the ATPase domain and association with other factors in this targeting pathway. In the apo (or ADP-bound) state, the two α-helical domains are separated from each other and folded in a manner that does not expose appreciable hydrophobic surfaces (Figure 3B). Upon ATP binding, the α-helical domains move together and re-configure to expose a large, methionine-rich, hydrophobic groove [48] that accommodates substrate TMDs [53].

TRC40/Get3 alone is not effective at capturing tail-anchored proteins in the cytosol [54,55], despite cytosolic ATP favouring the substrate-binding conformation [48,50]. Only when TRC40/Get3 is in a substrate-loading complex [56] can it efficiently acquire substrates from the chaperone SGTA (Sgt2 in yeast) [53–55,57,58]. This suggests that even in the ATP-bound state, TRC40/Get3 may be auto-inhibited. Interestingly, an amphipathic helix in each α-helical domain (orange in Figure 3B) was not visualized in crystal structures of the substrate-binding conformation of Get3 [48,53]. It is attractive to posit that this helix binds dynamically in the hydrophobic groove to inhibit promiscuous interactions. Thus, like SRP's M domain, TRC40's α-helical domains can form a methionine-rich hydrophobic groove that might be auto-inhibited by an intramolecular, amphipathic helix under normal circumstances. This model would explain the observed hydrophobicity threshold for substrates [54,59] and provide a rationale for why substrate binding is only favoured when substrate is presented at high local concentration: at the ribosome exit tunnel for SRP and at the substrate-loading complex for TRC40. The auto-inhibitory helix in both cases might serve as a lid in the substrate-bound conformation. We speculate that, although structurally unrelated, similar principles of substrate recognition have evolved in the two main, cytosolic TMD-binding targeting factors (Figure 3A,B).

General TMD-binding Factors: SGTA and Calmodulin

SGTA and Calmodulin are general TMD-binding factors that do not directly mediate either targeting or degradation of membrane proteins. Instead, they serve to generically protect TMDs from aggregation or inappropriate interactions in the cytosol. Clients dynamically interact with these factors until they engage either the membrane or load onto a targeting factor.

SGTA has been considered the most upstream factor in the tail-anchored targeting pathway to the ER [54,58]. However, it is not specific for only ER-destined tail-anchored proteins [54,60], and the effects of its depletion in vitro or deletion in yeast are very mild and selective [58,61]. Instead, SGTA is best viewed as a broad-specificity factor capable of engaging TMDs in a dynamic manner. The SGTA substrate complex can transiently sample the substrate-loading complex for TRC40 [53,54,57–59] (Figure 4A). If the SGTA-bound TMD is sufficiently hydrophobic, it can transfer directly and rapidly to TRC40 for ER targeting. If not, substrates can dissociate from SGTA to attempt engagement of machinery for mitochondrial insertion [60], ER insertion [62], or if these fail, degradation machinery [58]. Thus, SGTA is a general TMD-binding protein that precludes substrate aggregation and queries suitability for TRC40 loading. The mechanism of substrate binding by SGTA remains unclear, but it is noteworthy that the binding domain is methionine-rich, similar to the TMD-binding domains of TRC40 and SRP.

Calmodulin, which also binds its substrates via a flexible methionine-rich alpha-helical scaffold, has exceptionally broad specificity for a range of peptide sequences [63]. At physiological Ca^{2+} levels in cytosol, calmodulin dynamically engages both signal peptides and TMDs, particularly those of more modest hydrophobicity [19,62,64]. Structures of calmodulin with various peptide substrates [63,65] suggest that the bi-lobed calmodulin can effectively wrap around a hydrophobic domain to fully protect it from the aqueous environment (Figure 3C). The length of hydrophobic segments that can be shielded by calmodulin (up to ~18 residues) is appreciably longer than that favoured by protein folding factors such as Hsp70 [66]. This presumably explains why the latter is used for folding of soluble proteins, in which exposed hydrophobicity patches are typically only three to five residues, whereas the former engages signals and TMDs. Given the very high abundance of calmodulin in the cytosol and its dynamic interaction across the broad range of physiologic Ca^{2+} concentrations [19,62], it is effectively a buffer against signal-sequence and TMD aggregation. Unlike SGTA, however, calmodulin does not appear to directly interact with dedicated targeting factors. Thus, calmodulin should be considered a non-committed factor permissive for protein targeting, but not directly involved in the process.

Quality Control Factors: Ubiquilins and Bag6

In addition to targeting-specific and general TMD-binding factors, cells also contain TMD-binding factors that are linked to the protein ubiquitination machinery (Figure 4A). The first factor discovered to have this role was Bag6, which contains an amino-terminal ubiquitin-like (UBL) domain that recruits the E3 ubiquitin ligase RNF126 to mediate substrate ubiquitination and proteasomal degradation [58,67,68]. In the context of complete cytosol, Bag6 appears to have specificity for the long hydrophobic sequences that characterize TMDs. This interaction has a very slow off rate, making it effectively irreversible over physiological time frames [58]. The structural basis of Bag6 interaction with TMDs is not known, and unlike all of the other cytosolic TMD-binding factors, Bag6 does not contain a region of unusually high methionine content.

Remarkably, the carboxy-terminal region of Bag6 is an essential structural component of the TRC40 substrate-loading complex [58,69]. This part of Bag6 links the proteins Ubl4A, which recruits SGTA to this complex, and TRC35, which recruits TRC40 (Figure 4A). This core complex, lacking the TMD-binding and UBL domains of Bag6, is fully functional for tail-anchored protein loading onto TRC40. Bag6 is not present in yeast, where the homologs of Ubl4A and TRC35 (termed Get5 and Get4, respectively) interact directly in the Get3 substrate-loading complex [53,54,56]. It therefore appears that Bag6 is an embellishment embedded within the tail-anchored-protein pathway to route failed targeting substrates for degradation. The broader specificity of Bag6 for non-tail-anchored membrane proteins [67,68] and other particularly hydrophobic elements [68,70] makes it a general quality-control factor for most mislocalized membrane proteins.

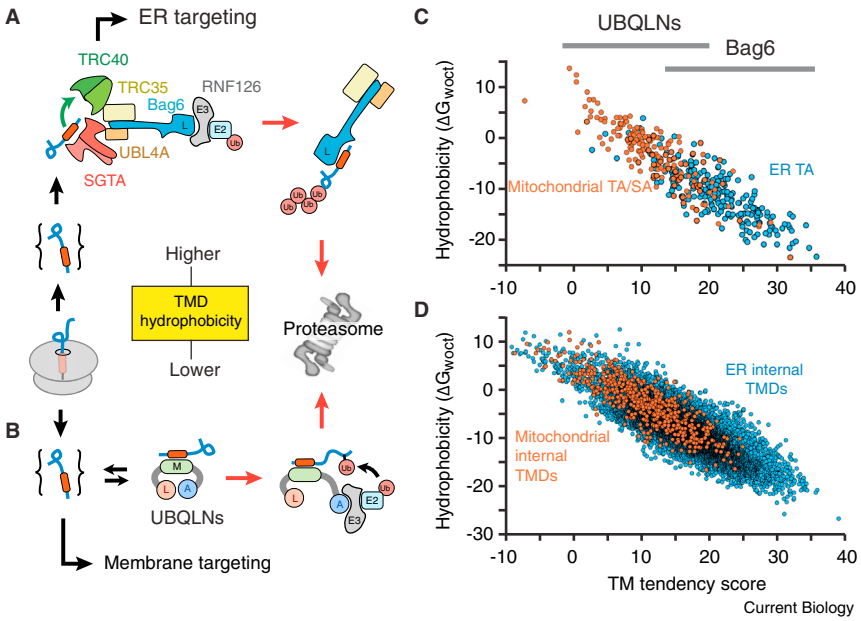

Figure 4. Quality control of mislocalized membrane proteins in the cytosol.
(A) Nascent chains containing a TMD (orange) released from the ribosome into the cytosol (in the lower left of panel A) can engage one of two quality control factors depending on TMD hydrophobicity. Those of higher hydrophobicity engage the Bag6 complex. Although this can occur directly (not shown), it typically occurs via an intermediate complex with the general TMD-binding factor SGTA. The SGTA–substrate complex is recruited to the Bag6 complex via a SGTA–UBL4A interaction, where substrate has an opportunity to transfer to TRC40 (recruited there by its interaction with TRC35). If transfer cannot or does not occur, substrates dissociate from SGTA and can be captured by Bag6. Substrate binding to TRC40 is a commitment to ER targeting, whereas binding to Bag6 is a commitment to degradation. The UBL domain of Bag6 ("L" in the figure) recruits the E3 ligase RNF126 for substrate ubiquitination (Ub) before targeting to the proteasome. (B) Substrates of lower hydrophobicity, characteristic of many mitochondrial TMDs, engage Ubiquilins (UBQLNs), which bind dynamically (via the "M" domain) to allow attempts at membrane insertion. The UBA domain ("A") of UBQLNs can recruit an E3 ubiquitin ligase that ubiquitinates substrates. This results in UBA interaction with substrate ubiquitin, exposing the UBL ("L") domain, which mediates targeting to the proteasome for degradation. (C) The TMDs of membrane proteins post-translationally targeted to the ER (blue) or mitochondria (orange) were plotted on the basis of their hydrophobicity according to two scales: TM tendency (x-axis) as defined by Zhao and London [7], and ΔG_{woct} (y-axis) as defined by White and Wimley [147]. TMDs that mediate targeting to mitochondria are systematically less hydrophobic by either scale than TMDs targeted to the ER. UBQLNs are thought to favour mitochondrial TMD substrates, while Bag6 engages ER TMDs. TA, tail anchor; SA, signal anchor. (D) The internal (that is, non-targeting) TMDs of all ER and mitochondrial membrane proteins were plotted as in panel C. Note that among internal TMDs, the spread of hydrophobicity is very broad, with little difference between the ER and mitochondria.

In addition to membrane proteins mislocalized to the cytosol, Bag6 also recognizes membrane proteins dislocated into the cytosol from the ER membrane during ER-associated degradation [71–73]. In this function, a sub-population of Bag6 is recruited to the site of dislocation [74], where it presumably captures hydrophobic domains as they emerge into the cytosol. As with mislocalized proteins, Bag6 acts to prevent aggregation, and presumably ferries the substrate to the proteasome. Hence, in its absence, substrates dislocated during ER-associated degradation will aggregate in the cytosol. Thus, Bag6 appears to patrol the cytosol for membrane proteins, regardless of their source, and ensures their degradation before they can aggregate. SGTA may similarly help to prevent aggregation during ER-associated degradation [75], presumably by shielding exposed hydrophobic segments in conjunction with Bag6.

More recently, the Ubiquilins were discovered as a family of TMD-binding factors that can couple with the protein ubiquitination machinery for substrate degradation [60]. Four Ubiquilins (UBQLN1 to UBQLN4) are found in mammals, and the sole yeast homolog is known as Dsk2. Biochemical studies show that unlike Bag6, Ubiquilins engage substrates dynamically [60]; hence, substrate binding to Ubiquilins is not a commitment to degradation (Figure 4A). In this manner, Ubiquilins can preclude membrane protein aggregation and, for an initial period of time, allow opportunities for engagement of insertion machinery at a target membrane (which, in the case of many Ubiquilin clients, is the mitochondrial outer membrane). Over time, however, Ubiquilins recruit a yet-unidentified E3 ligase to mediate substrate ubiquitination. This is the commitment step for degradation because substrate ubiquitin binds to a ubiquitin-associating domain in Ubiquilin, preventing substrate dissociation while simultaneously favouring proteasome targeting via a ubiquitin-like domain in Ubiquilin [60].

The TMD specificity of Ubiquilins appears to be roughly similar to that of Bag6, but with a preference for lower hydrophobicity [60]. This is consistent with its binding to mitochondrial membrane proteins, whose TMDs are typically less hydrophobic than those destined for the ER (see, for example, Figure 4B). Although the substrate-binding region of Ubiquilins remains to be investigated structurally, it is noteworthy that — like the substrate binding regions of SGTA [54], TRC40 [48], calmodulin [65], and SRP [26] — Ubiquilins are also rich in methionine.

Cultured cells lacking Ubiquilins show impaired degradation of mitochondrial membrane protein precursors that fail to import [60]. Stimulated B-cells experiencing mitochondrial stress are acutely dependent on UBQLN1, the absence of which leads to mitochondrial precursor accumulation and cell death [76]. Different Ubiquilin family members might have somewhat different substrate binding properties given that UBQLN4 seems to engage ER-destined membrane proteins that fail targeting [77]. Thus, in vitro and in vivo, Ubiquilins and Bag6 appear to monitor the cytosol for exposed TMDs to prevent aggregation and favour their degradation, a function that might be particularly important when protein import is impaired during organelle stress [76,78,79]. In addition to direct recognition of TMDs, Ubiquilins and Bag6 have been implicated in the turnover of a range of TMD-lacking proteins [71,80–83]. Whether such functional requirements for turnover reflect direct recognition of substrates, recognition of ubiquitin chains attached to the substrate, or recognition via a chaperone adaptor remains to be investigated in detail.

Relationships among Cytosolic TMD-binding Factors

The picture that emerges is that the cytosol has several abundant factors capable of recognizing TMDs and shielding them from solvent (Figure 1A,C). Most of these factors in isolation have remarkably broad substrate ranges for almost any hydrophobic region of a polypeptide. Although this overlap provides cells with multiple 'back-ups' to minimize unchaperoned TMDs, it raises the critical issue of how any degree of specificity is achieved. This is important because each TMD-binding factor couples to a different downstream factor that ultimately dictates substrate fate. Presumably, a combination of relative abundances, localization, subtle differences in specificity, and different on-rates of substrate binding determine which TMD-binding factor is initially favoured. Interactions among the different factors and different substrate off-rates would then determine how substrates partition among the factors over time.

For example, SRP's location at the ribosome exit tunnel (Figure 1B) gives it the highest priority for co-translational recognition, even though its overall low abundance and auto-inhibition places it last in line for post-translational recognition. Recent analysis of mammalian tail-anchored-protein pathway factors suggests that a combination of abundance and relative on-rates favour initial SGTA engagement over either Bag6 or TRC40 [58]. Substrates can transfer from SGTA to either TRC40 or Bag6, with TRC40 being both faster and higher priority. Once substrate is bound to TRC40 or Bag6, its very slow rate of dissociation from either factor effectively commits it to ER targeting or proteasomal degradation, respectively. Analogous kinetic analysis of substrate flux through the various TMD-binding factors at their appropriate relative concentrations will ultimately be needed to explain how they triage nascent membrane proteins between different potential fates to maintain cytosolic protein homeostasis.

Membrane Factors for TMD Recognition

Maintaining the solubility of a membrane protein until it encounters the ER (or other organelle) is only the first requirement for membrane protein insertion. The second essential step is the actual insertion reaction. This does not occur unassisted, but requires a factor that, at minimum, recognizes TMDs and provides access to the interior of the lipid bilayer. Membrane insertion factors therefore need to help hydrophobic TMDs overcome the hydrophilic barrier imposed by the polar head groups of phospholipids. Quality-control factors in the membrane must also recognize TMDs, but facilitate the reverse reaction of dislocating a TMD from the lipid bilayer for degradation in the cytosol. The different TMD-recognition factors at the membrane may use similar core principles to provide a gated route between the aqueous cytosol and hydrophobic lipid bilayer.

The Sec Translocation Channel

The best understood route for membrane protein insertion is the universally conserved Sec translocon, which also mediates soluble-protein translocation [84]. Whereas the Sec translocation channel can operate in both post- and co-translational modes, only the latter mode is used for membrane proteins. Ribosome–nascent-chain complexes are delivered to the Sec translocon by the SRP pathway. The ribosome engages the Sec translocon such that the ribosome's exit tunnel is aligned with the translocon's central pore [85]. This arrangement allows new TMDs emerging from the ribosome to engage the translocon without significant exposure to the cytosol. Co-translational recognition and insertion of individual TMDs in succession obviates the challenge of maintaining the solubility and insertion competence of a highly hydrophobic, complicated, multi-spanning membrane protein. Hence, essentially all multi-pass membrane proteins are thought to use the Sec translocon.

The central component of the Sec translocon is the three-protein Sec61 complex in eukaryotes, and the homologous SecY complex in prokaryotes and archaea. Biochemical studies had long established that Sec61 not only lines a pore across the membrane [86], but also recognizes hydrophobic domains [87] and provides them lateral access to the lipid bilayer [88–90]. These biochemical insights helped rationalize and interpret ensuing structures of the Sec translocon. The seminal X-ray structure of the isolated archaeal SecY complex showed that a single SecY (Sec61α in eukaryotes) forms a pseudo-symmetrical clamshell surrounding an hourglass-shaped central pore [91] (Figure 5A). The narrowest part of this pore is lined by a ring of six conserved hydrophobic residues, atop which sits a short plug helix that occludes the channel. The back of the clamshell is braced by SecE (Sec61γ in eukaryotes), whereas Secβ (SecG in bacteria and Sec61β in eukaryotes) is more peripheral. It was postulated that the plug helix would be displaced when the channel opens, and the seam at the front of the clamshell could act as a lateral gate between the central pore and lipid bilayer.

As with SRP, major advances in single-particle cryo-electron microscopy have permitted native ribosome–Sec61 complexes, previously analysed at lower resolution [92,93], to now be visualized at close to atomic resolution [94–96]. In parallel, advances in cryo-tomography now permit sub-nanometer views of the translocon in the native membrane environment [97]. Mechanistic insight into the recognition of substrates (signal sequences and TMDs) by Sec61α has come from structural comparisons of Sec61α (or SecY) before and after substrate engagement. Relative to the quiescent SecY crystal structure [91], ribosome-bound (but substrate-free) Sec61 complex shows several small conformational changes that 'prime' the channel for subsequent substrate recognition [96]. Ribosome binding constrains two cytosolic loops in the carboxy-terminal half of Sec61α, thereby causing subtle shifts in their associated transmembrane helices. This movement is propagated through to the remaining transmembrane helices, leading to a cracked lateral gate awaiting the arrival of substrate (Figure 5B).

The structure of a signal sequence-engaged ribosome–Sec61 complex revealed additional conformational changes that accompany substrate binding (Figure 5B). As expected from earlier photo-crosslinking experiments [88–90], the substrate forms an alpha helix positioned at the Sec61α lateral gate and exposed to surrounding lipid. To accommodate substrate at this site, the amino-terminal half of Sec61α makes a simple 22° rigid-body rotation hinged at the back of the clamshell and parting the lumenal portion of the lateral gate. The carboxy-terminal half of Sec61α remains in place relative to the ribosome, to which it is tightly anchored. Widening of the lateral gate causes the central pore to widen, destabilizing the plug's position there. In this way, substrate recognition is coupled to channel opening, resulting in a conformation in which the aqueous pore and lipid bilayer are connected via the lateral gate, where the signal

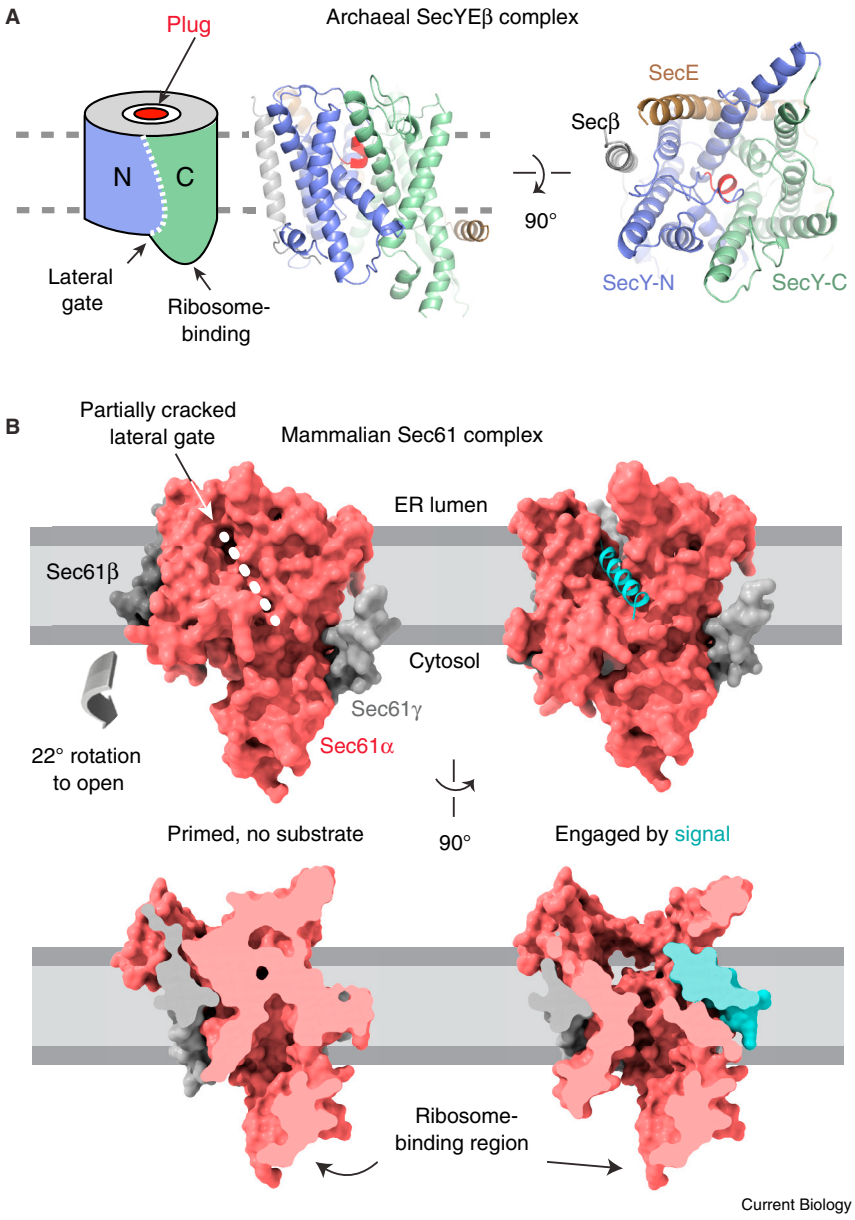

Figure 5. Hydrophobic domain recognition by the Sec translocon.
(A) Overview of the Sec translocon architecture based on the crystal structure of the archaeal SecYEβ complex (PDB code 1RH5). Left diagram shows the key features evident in the structures shown at right, with N and C representing the amino-terminal and carboxy-terminal halves of the pseudo-symmetrical SecY protein. SecE and Secβ are omitted from the diagram for simplicity. (B) Structures of the ribosome-bound mammalian Sec61 complex (PDB codes 3J7Q and 3JC2) before (left) and after (right) binding of a hydrophobic signal peptide (cyan). The alpha, beta, and gamma subunits of the Sec61 complex are in red, dark grey, and light grey, respectively. The position of the lateral gate, which is partially cracked by ribosome binding, is indicated by the dashed line. Binding of the signal peptide is accompanied by a ~22° rotation of the amino-terminal half of Sec61α to open the lumenal half of the lateral gate, where the signal peptide binds. The lower images represent cutaways at the plane of the central pore after 90° rotation of the structures shown above. Note in the cutaways that, without a bound signal, the channel is closed toward both the lipid bilayer and across the membrane. Signal binding results in a continuous pore across the membrane, with different regions of the signal peptide accessible to the central pore and lipid bilayer.

peptide is bound (Figure 5B). The same overall architecture was observed in a crystal structure of SecY bound to a hydrophobic peptide fused to the SecY partner SecA [98], and in Sec61 complex in native ER membranes apparently bound to endogenous substrates [97,99]. Thus, the mechanism of substrate recognition is highly conserved and occurs similarly across species and in the native membrane environment of the ER.

Remarkably, the engaged signal sequence occupies the position previously held by transmembrane helix 2 of the lateral gate, effectively taking over its former interactions with helix 7. It is attractive to postulate that only substrates capable of displacing helix 2 are effectively recognized. In this model, the hydrophobicity threshold for substrate recognition is set by the properties of an internal helix in Sec61α, analogous to signal recognition by SRP. Consistent with this view, strengthening or weakening interactions of the lateral gate that interface with either mutations [100] or small molecules [101] affects the hydrophobicity threshold of substrate recognition.

Future structural analysis of Sec61 engaged with biophysically diverse signals and TMDs will be needed to understand how heterogeneous endogenous substrates gain access to the membrane. This is particularly important in the context of multi-pass membrane protein insertion. The prevailing model posits that TMDs are individually and successively recognized by the translocon and integrated into the ER as they emerge from the ribosome [102,103]. However, multi-pass membrane proteins contain TMDs with a wide range of hydrophobicities (Figure 4C), and many of these are remarkably hydrophilic. Indeed, these internal TMDs are often not recognized by Sec61 when tested in isolation [104]. It is likely that interactions between such 'weak' TMDs and other TMDs within the same protein [105–107], or even provided in trans [108], facilitate insertion. How such cooperation might occur, and the role of Sec61 or other, poorly studied accessory factors [109,110], remains an important direction for future study.

TMD Insertases

Many membrane proteins are inserted into the lipid bilayer by Sec-independent mechanisms. Although this reaction can occur spontaneously under certain experimental conditions [111,112], it is typically mediated by factors generically termed 'insertases'. The first non-Sec insertases were defined in the bacterial plasma membrane [113] and the topologically

equivalent inner-mitochondrial [114] and chloroplast membranes [115]. The bacterial (YidC), mitochondrial (Oxa1), and chloroplast (Alb3) insertases are evolutionarily related to each other, whereas the ER membrane houses three seemingly unrelated insertases termed the Get1/2 complex, the ER membrane-protein complex (EMC), and TMCO1. As discussed later in this section, recent analyses indicate that all of these factors may have arisen from a common ancestor [116], suggesting that they could share similar mechanisms of action.

The requirement for an insertase in the ER was first suggested by the need to insert tail-anchored proteins targeted to the membrane by TRC40 [43]. The receptor for this targeting factor also proved to be the insertase, and is composed of the heterodimeric Get1/Get2 complex (WRB/CAML in higher eukaryotes). Get1 and Get2 are both ER-localized, three-TMD membrane proteins. They were originally identified as a putative receptor for the targeting factor Get3 based on genetic and physical interaction studies in budding yeast [45], and then demonstrated in reconstitution studies with recombinant factors to constitute the minimal insertion machinery [117]. Single-molecule analysis suggests that a single Get1/2 heterodimer is sufficient for insertion of tail-anchored proteins into the lipid bilayer [118].

A mechanistic model for TMD transfer from Get3 to the Get1/2 complex has been deduced from structural analysis [117,119] and accompanying biochemical studies [117,120]. A short two-helix motif at the end of Get2's flexible amino-terminal cytosolic tail initially recruits the intact Get3–TMD complex to the membrane. Once at the membrane, a cytosolic coiled-coil in Get1 wedges between the two alpha-helical domains of the Get3 dimer. This interaction necessarily disrupts the hydrophobic groove formed by the previously juxtaposed alpha-helical domains, thereby inducing substrate release. Because the tip of Get1's coiled-coil protrudes into the ATP-binding site of Get3, ATP hydrolysis (or dissociation) must occur before Get1 can act; hence an ATP-hydrolysis mutant of Get3 cannot effectively release substrate and acts as a dominant-negative factor in vitro [43]. Conversely, re-binding of ATP to the substrate-free Get3–Get1 complex promotes recycling of Get3 back to the cytosol.

The mechanism of TMD insertion by the Get1/2 complex is not known, but it appears to involve the membrane domains of Get1 and Get2 [120]. Hence, tail-anchored-protein insertion is impaired by mutations in the Get1/2 membrane domains, which also physically crosslink tail-anchored-protein insertion intermediates. These observations imply that the Get1/2 complex recognizes TMDs and provides a path from the cytosol into the membrane. Structures of the Get1/2 complex, together with structure-guided mutagenesis, will be needed to understand the mechanism of its insertase activity.

It has been known since the discovery of TRC40 that not every tail-anchored protein can effectively engage this factor [43]. This conclusion was reinforced in the ensuing years by the partial tail-anchored-insertion defects seen in yeast lacking Get-pathway factors [45,61], and incomplete insertion defects in TRC40-deficient lysates, cells, and tissues [58,121,122]. Recent analysis of tail-anchored-protein insertion in vitro showed that TRC40 preferentially favours the most hydrophobic TMDs [62]. Those of moderate and low hydrophobicity neither engage TRC40 stably, nor use the WRB/CAML receptor. Instead, these tail-anchored proteins depend on EMC [62], a 10-protein highly conserved resident ER factor of previously unknown function [61]. Disruption of EMC strongly impairs insertion of the low- and moderate-hydrophobicity tail-anchored proteins in vitro and in cultured cells, whereas purified EMC in synthetic liposomes was sufficient to mediate near-native insertion efficiency in vitro. Analysis of tail-anchored proteins spanning the natural range of hydrophobicities suggests that in a general sense, EMC deals with the lower half, while TRC40 is used by the upper half [62].

EMC would at first seem entirely unrelated to the Get1/2 insertase. However, homology searches of Get1 using HHpred [123] identifies the EMC3 subunit of EMC with high confidence, and both appear to be members of the DUF106 family. The crystal structure of an archaeal DUF106 family member (Mj0480) revealed a three-TMD protein that structurally aligns well with three of the five TMDs of the bacterial insertase YidC [124]. Indeed, YidC, Oxa1, and Alb3, along with a resident ER protein called TMCO1, are among the top high confidence sequences identified in HHpred searches with Get1, EMC3, or Mj0480 [116]. Both Mj0480 and TMCO1 were observed in photo-crosslinking assays to interact with a nascent TMD-containing protein [116,124], although native substrates for either factor remain to be identified. These findings collectively argue for an ancient evolutionary relationship between the prokaryotic YidC family of insertases and the eukaryotic ER insertases Get1/2, EMC, and TMCO1, suggesting that they might share similarities in core structure and mechanism of TMD insertion.

The structure of YidC [125] showed that it contains five TMDs arranged to form a partially hydrophilic groove that is open towards both the lipid bilayer and the cytosol (Figure 6A). Crosslinking studies suggest this groove operates as a binding site for TMDs [126], which are then released into the membrane. The absence of a membrane-spanning channel within YidC is consistent with its inability to handle membrane proteins that require translocation of a soluble domain across the lipid bilayer. Structural information about the Get1/2 complex, EMC, and TMCO1 will be needed to determine the extent to which these factors operate similarly to YidC. Furthermore, YidC is thought to additionally use its TMD-binding activity to act as a membrane protein chaperone in conjunction with the Sec translocon [127–129]. Whether any of the analogous ER complexes have this capacity remains to be determined. Among them, EMC is noteworthy as it has been indirectly implicated in several, as yet poorly understood, aspects of the biogenesis, trafficking, or degradation of several membrane proteins [130–132]. Whether these effects are direct, or a consequence of failed tail-anchored-protein insertion, remains to be investigated.

Quality Control Factors: the Hrd1 and Derlin Families

Whereas Sec61, Get1/2, and EMC all recognize TMDs for the purpose of insertion, the ER contains other TMD-recognition factors that participate in quality control and degradation. ER quality-control machinery ensures that only correctly folded proteins are trafficked to their final destinations [133]; the misfolded proteins are routed for degradation via pathways of ER-associated degradation [134]. When misfolding occurs within the membrane-localized section of a membrane protein, the TMDs must ultimately be recognized and extracted out of the lipid bilayer for degradation by the proteasome. The factors that mediate this recognition are incompletely defined, but include

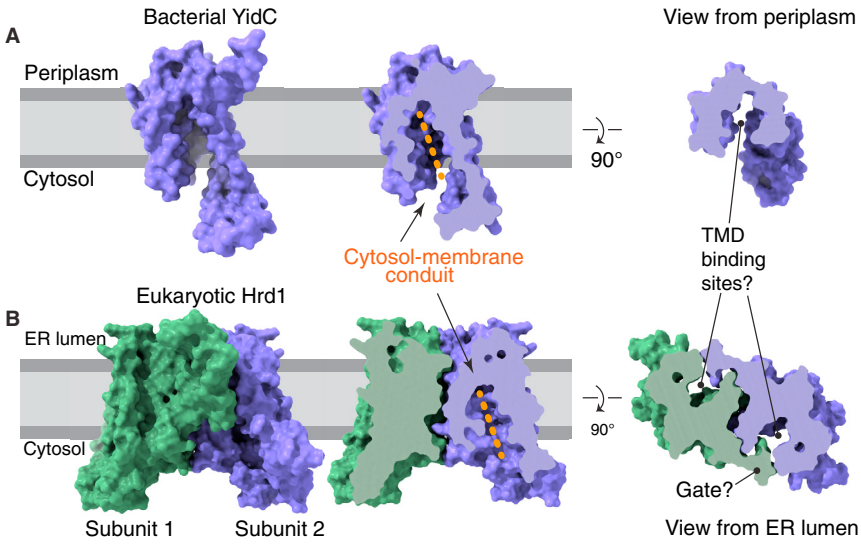

Figure 6. Insertases and dislocases may share similar architectural features.
Structures of the bacterial insertase YidC (panel A; PDB code 3WO6) and the homo-dimeric yeast Hrd1 dislocation channel (panel B; PDB code 5V6P). The conduits that potentially connect the cytosol with the membrane are indicated by orange dashed lines. The right diagrams show 90°-rotated structures cut in the plane of the membrane. The proposed regions of TMD binding are indicated. In the case of Hrd1, this putative binding site is occluded by the neighbouring subunit, perhaps serving as a gate to control access.

the conserved Hrd1 and Derlin families. Recognition of misfolding on the lumenal and cytosolic faces of the ER membrane involves yet additional factors that are not considered here.

Hrd1, initially discovered as a factor for regulated degradation of HMG-CoA reductase [135], is a highly conserved membrane protein with a cytosolic E3-ligase domain. Analogous to Sec61 in forward translocation, Hrd1 is thought to form a channel across the membrane for retro-translocation [136,137], while also recognizing TMDs within the lipid bilayer [138]. A recent cryo-electron microscopy structure of Hrd1 in complex with its tightly bound partner Hrd3 showed that Hrd1 exists as a dimer, with each molecule containing eight transmembrane segments [139]. Five of these helices form a cytosolically exposed aqueous cavity that spans more than halfway across the membrane (Figure 6B), reminiscent of YidC (Figure 6A). The cavity is sealed laterally by TMD1 from a neighbouring Hrd1 molecule and lumenally by a two-layer seal of hydrophobic residues. Although the functional state represented by this structure is currently uncertain, it is attractive to posit that substrate TMDs can engage Hrd1 through a lateral gate occupied by TMD1. The hydrophilic features of the aqueous cavity might favour recognition of partially hydrophilic TMDs, exposure of which could be a cue to infer misfolding or mis-assembly.

In addition to Hrd1, some misfolded substrates require a Derlin family member for degradation [140,141]. Derlins are ER-membrane proteins containing six TMDs and are inactive members of the Rhomboid superfamily of intramembrane proteases [142]. To cleave their substrates, Rhomboids must be capable of selective TMD recognition; analogously, inactive Rhomboids also have TMD-recognition capacity for the purposes of modulating client trafficking and turnover [143]. It is therefore likely that Derlins have adapted this property to recognize TMDs of misfolded proteins and deliver them to Hrd1 for subsequent dislocation. This is an attractive model because the multiple Derlin family members (three in mammals), together with Hrd1 and other membrane-embedded E3 ligases [144,145], would each have differing specificity to collectively recognize the diversity of possible degradation substrates. The structural basis for how Derlins distinguish normal from aberrant TMDs requires future investigation.

Future Directions

Four general areas merit priority for understanding membrane protein homeostasis. First, it is imperative that we have a full accounting of all cytosolic and membrane-embedded TMD-recognition factors. The topologic and biophysical diversity of membrane proteins means their recognition is an insurmountable task for any one factor. Indeed, the topologic constraints of SRP [146] pointed the way to TRC40 [43], and the biophysical constraints of the TRC40 pathway revealed the insertase function of EMC [62]. As more complex and diverse substrates are examined in depth, the need for, and identity of, additional factors may be revealed.

Second, the client range for most factors remains to be defined. Due to pathway redundancy, compensation, and indirect consequences, defining clients by analysis of factor-deleted cells can be misleading. Instead, direct measurement of flux under normal homeostatic conditions is needed. While the physical coupling of SRP to the mRNA of its clients permitted deep-sequencing methods to be employed [17,18], this approach is unsuited to post-translationally acting factors with fleeting substrate interactions. It is hoped that increasingly sensitive mass spectrometry combined with rapid proximity-based labelling methods might provide one viable route.

Third, the molecular and structural basis of substrate recognition is unknown for many factors. TMD-containing nascent protein substrates are challenging to biochemically manipulate, trap on their recognition factors, and isolate in large quantities. Although this has been a major impediment to crystallography, cryo-electron microscopy now affords new opportunities for the analysis of low-abundance and heterogeneous samples across a broad size range. This will undoubtedly be a major boon to understanding the common principles of TMD recognition, particularly by membrane factors about which we currently know little.

Finally, the inter-relationships between the different TMD-recognition factors and the basis of their selectivity remain to be defined. It is increasingly clear that in the absence of competition in vitro, chaperone-like proteins can be highly promiscuous, engaging many substrates they ordinarily never bind under native conditions. Although one must necessarily use highly purified systems to investigate molecular mechanisms, assignment

of physiological roles by simple extrapolation can be problematic. Thus, investigation of TMD-recognition factors in increasingly complex and native-like experimental systems will be necessary to understand how fate decisions are made. This is undoubtedly a critical issue because both promiscuous quality control and excessive biosynthetic attempts can perturb protein homeostasis and lead to diseases.

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