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The Biogenesis of Multipass Membrane Proteins

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Multipass membrane proteins contain two or more α-helical transmembrane domains (TMDs) that span the lipid bilayer. They are inserted cotranslationally into the prokaryotic plasma membrane or eukaryotic endoplasmic reticulum membrane. The Sec61 complex (SecY complex in prokaryotes) provides a ribosome docking site, houses a channel across the membrane, and contains a lateral gate that opens toward the lipid bilayer. Model multipass proteins can be stitched into the membrane by iteratively using Sec61's lateral gate for TMD insertion and its central pore for translocation of flanking domains. Native multipass proteins, with their diverse TMDs and complex topologies, often also rely on members of the Oxa1 family of translocation factors, the PAT complex chaperone, and other poorly understood factors. Here, we discuss the mechanisms of TMD insertion, highlight the limitations of an iterative insertion model, and propose a new hypothesis for multipass membrane protein biogenesis based on recent findings.

Integral membrane proteins participate in a diverse range of biological processes, including transmembrane signaling, nutrient transport, ion homeostasis, membrane trafficking, and others (von Heijne 2007). To serve these functions, membrane proteins must be inserted into the lipid bilayer in a defined orientation, fold into a functional state, and be localized to the correct part of the cell. Except for β barrels, all integral membrane proteins contain at least one α-helical transmembrane domain (TMD) and are the subject of this review. Almost all membrane proteins are initially inserted into the endoplasmic reticulum (ER) membrane in eukaryotes and plasma membrane in prokaryotes (Rapoport et al. 2017; Hegde and Keenan 2022). Because these membranes are evolutionarily related to each other, the core machinery for membrane protein biogenesis is widely conserved across all organisms.

The universal steps of membrane protein biogenesis involve two separable reactions (Fig. 1). The first is nascent protein targeting to the membrane. The second is TMD insertion into the lipid bilayer concomitant with translocation of a flanking polypeptide domain across the membrane. The targeting step typically occurs cotranslationally and is mediated by the signal-recognition particle (SRP) and its membrane-localized receptor (Akopian et al. 2013). The translocation reaction that accompanies TMD insertion is mediated by one or both of two universal protein translocation factor families: SecY (Sec61 in eukaryotes) or Oxa1 (various names in different systems, as described later; see Fig. 1 legend). The SRP system and both translocation factors existed in the last universal common ancestor and are conserved.

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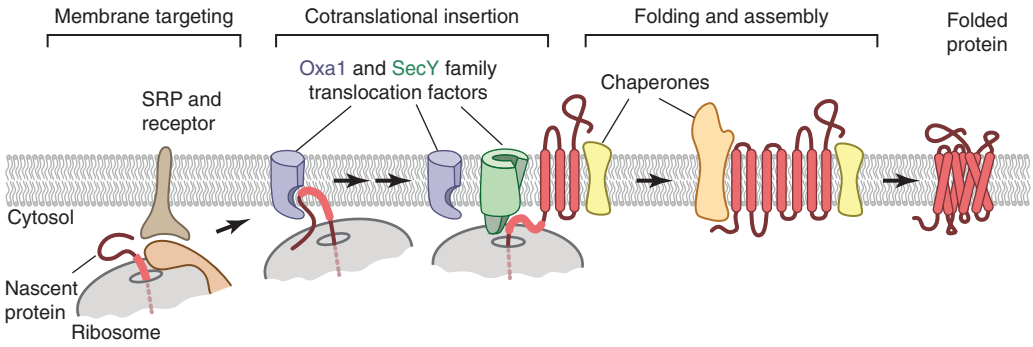

Figure 1. Overview of membrane protein insertion. Membrane proteins are targeted to the prokaryotic plasma membrane or eukaryotic endoplasmic reticulum (ER) by signal-recognition particle (SRP) and its membrane-localized receptor. SRP recognizes the first hydrophobic domain (in this example, a transmembrane domain [TMD]) as it emerges from the ribosome exit tunnel. After targeting, TMDs are inserted by members of the Oxa1 family, SecY family, or both. Putative chaperones can promote membrane protein folding and assembly by preventing inappropriate interactions and premature degradation. The founding member of the Oxa1 family is Oxa1 of the mitochondrial inner membrane. Homologs are found in the bacterial plasma membrane (YidC), archaeal plasma membrane (Ylp1, also known as MJ0480 in *Methanocaldococcus jannaschii*), chloroplast thylakoid membrane (Alb3), and the ER membrane (EMC3, Get1, and TMCO1). All of these membranes are evolutionarily related. Ylp1, EMC3, Get1, and TMCO1 are thought to have a conserved binding partner (MJ0606, EMC6, Get2, and C20Orf24, respectively), whereas YidC, Oxa1, and Alb3 do not. SecY is the prokaryotic name for this family member. Its homolog in the yeast ER is known as Sec61 and its homolog in the mammalian ER is Sec61α. All of these are part of heterotrimeric complexes with two small subunits (SecE and SecG in bacteria, Sbh1 and Sss1 in yeast, and Sec61β and Sec61γ in mammals).

SecY family members contain a membrane-spanning channel capable of translocating any length and composition of unfolded polypeptide across the membrane (Rapoport et al. 2017). SecY and Sec61α are part of heterotrimeric complexes with two small subunits termed SecE and SecG in *Escherichia coli*, and Sec61β and Sec61γ in mammals (Fig. 2A). The channel is formed at the interface of the two pseudosymmetric antiparallel halves of Sec61α (Van den Berg et al. 2004). The two halves can open toward the lipid bilayer like a clamshell. The front side of the clamshell is called the lateral gate and the back side is the hinge. The back is braced by the single diagonal TMD of Sec61γ, with Sec61β on one side. The central channel is occluded by a short plug helix that is displaced during polypeptide translocation.

In contrast to SecY, Oxa1 family members (Fig. 2B) contain a hydrophilic vestibule that penetrates only part of the way across the membrane—a half-channel (Kumazaki et al. 2014; Hennon et al. 2015). This vestibule is formed by a highly conserved three-TMD bundle (Anghel et al. 2017; McDowell et al. 2021). The three-TMD core forms a heterodimer with an obligate three-TMD partner in the eukaryotic ER and archaea (Hegde and Keenan 2022). In bacteria and bacteria-derived organelles (e.g., mitochondria and chloroplasts), the three-TMD core is supplemented with additional TMDs within the same protein. The vestibule facilitates translocation of short polypeptide segments (less than ~50 amino acids), perhaps by locally thinning the membrane barrier (Hennon et al. 2015; Wu and Rapoport 2021). The pseudosymmetric SecY channel might have originated by duplication and fusion of an Oxa1-like half-channel interacting in an antiparallel fashion (Lewis and Hegde 2021).

Many membrane proteins contain multiple TMDs that weave back and forth across the membrane. These multipass proteins constitute more than half of all membrane proteins in the human genome and have extensive roles in cell and organismal physiology (The Uni-

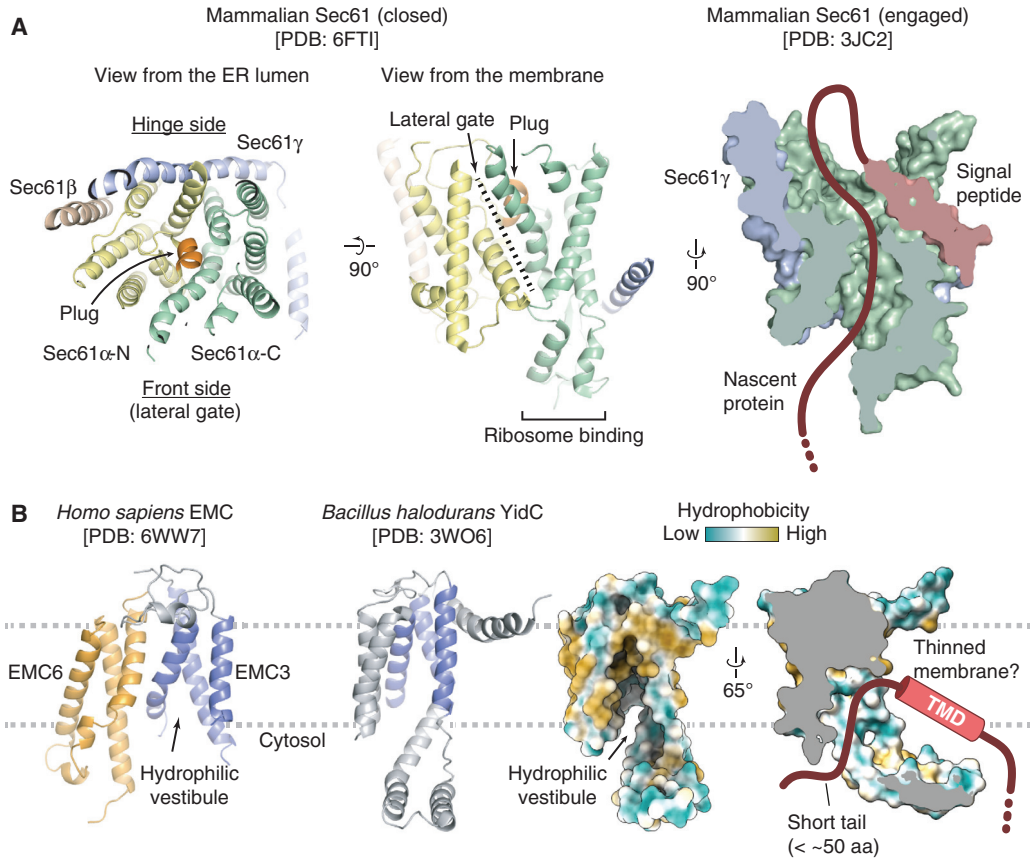

Figure 2. Structural features of SecY- and Oxa1-family translocation factors. (*A*) The mammalian Sec61 complex is shown in closed (*left* and *middle*) and engaged (*right*) states. The pseudosymmetric amino- and carboxy-terminal halves of Sec61α (SecY in prokaryotes) are colored in yellow and green, respectively, in the closed structure from PDB 6FTI (Braunger et al. 2018). The engaged structure (PDB 3JC2) contains a signal peptide at the lateral gate and a continuous channel across the membrane through which a nascent chain can pass (Voorhees and Hegde 2016). Archaeal and bacterial SecY complexes have very similar structures (Van den Berg et al. 2004; Li et al. 2016). (*B*) Structures of human EMC3 (Pleiner et al. 2020) and bacterial YidC (Kumazaki et al. 2014) as examples of Oxa1 family members that are part of heteromeric complexes or are monomeric, respectively. The signature three-transmembrane domain (TMD) core of the Oxa1 family is in blue. The membrane domain of the EMC3–EMC6 subcomplex shown represents the functional core of the nine-subunit ER membrane protein complex (EMC). The surface representations of YidC show the hydrophilicity of its vestibule, which facilitates flanking domain translocation during TMD insertion (*right*). The membrane might be locally distorted and thinner adjacent to the vestibule. The membrane domains of the Get1–Get2 complex (McDowell et al. 2020) and the predicted TMCO1–C20Orf24 complex (Lewis and Hegde 2021) are similar to the EMC3–EMC6 structure.

Prot Consortium 2018). Beyond the targeting and TMD insertion steps shared with single-TMD proteins, multipass proteins must then insert additional TMDs, which eventually pack together into the correct folded structure (Fig. 1). Multipass proteins vary widely in the number of TMDs, their sequences, biophysical properties, and spacing (White and von Heijne 2005; Guna and Hegde 2018). How such a diverse range of membrane proteins is inserted correctly into the membrane is the focus of this review.

THE INITIATION OF MULTIPASS PROTEIN BIOGENESIS

Multipass proteins are inserted into the ER membrane cotranslationally. In this section, we consider the steps up to insertion of the first N_{exo} TMD (that is, a TMD whose amino-terminal flanking domain is translocated to the exoplasmic side of the membrane). This initiation phase of multipass proteins (Fig. 3) is similar to the insertion of single-TMD proteins. There are three types of single-TMD proteins whose biogenesis is relevant for multipass proteins, with tail-anchored (TA) proteins forming a fourth type that is reviewed elsewhere (Chio et al. 2017). The types of single-TMD membrane proteins are classified by the orientation of the first TMD and mechanism of flanking hydrophilic domain translocation.

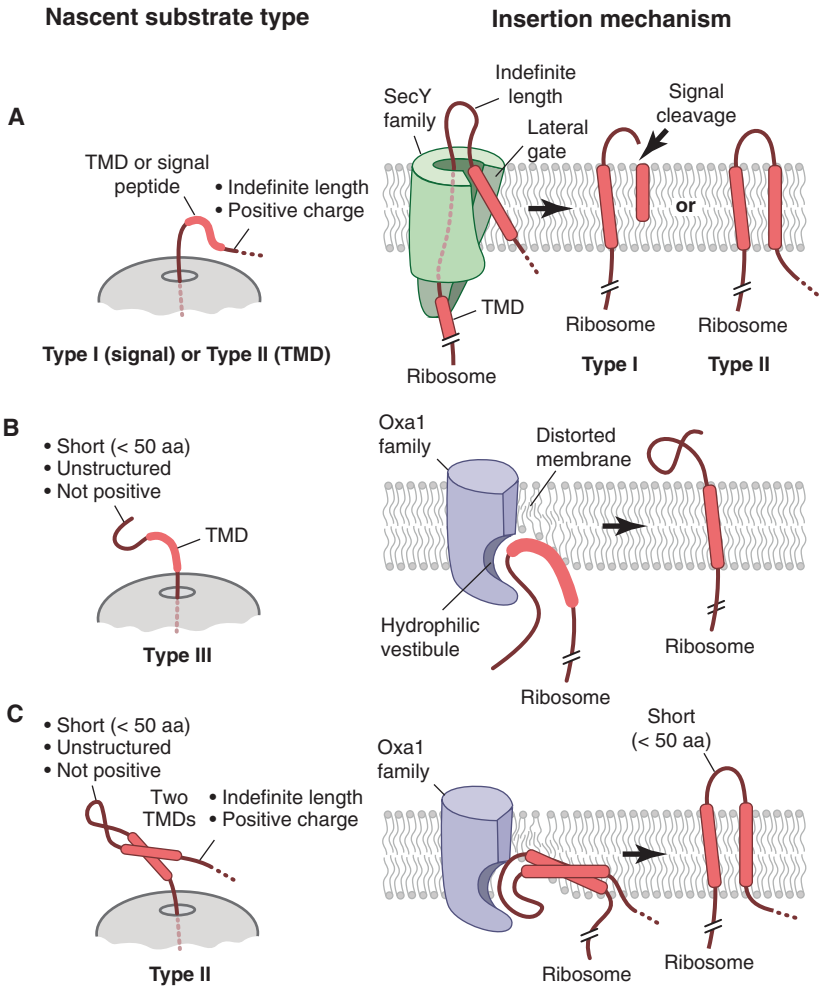

Figure 3. The initiation phase of multipass protein insertion. (*A*) Model for insertion of type I and type II membrane proteins by the SecY family. The substrate that is delivered to SecY is shown on the *left*, and the key insertion intermediate depicted on the *right*. (*B*) Insertion of type III membrane proteins by the Oxa1 family. (*C*) Insertion of type II proteins by the Oxa1 family. In very rare instances, it might be possible for type I proteins to use a similar mechanism (Celebi et al. 2006; Van Bloois et al. 2006), but with a cleavable signal and transmembrane domain (TMD) being inserted as a pair instead of two TMDs as depicted for type II proteins. Key features of the substrate for each situation are indicated.

Type I Membrane Proteins

Membrane proteins that have a translocated domain longer than ~50 amino acids preceding the first TMD (which will be in the N_{exo} topology) contain an amino-terminal cleavable signal peptide (Wallin and von Heijne 1995; The UniProt Consortium 2018). These are termed type I membrane proteins. SRP recognizes the hydrophobic signal peptide as it emerges from the exit tunnel of the ribosome and the ribosome-SRP complex is targeted to the SRP receptor (SR) at the membrane. In a handover reaction that is incompletely understood, the SRP–SR complex on the ribosome is exchanged for the Sec61 complex (Kobayashi et al. 2018; Jomaa et al. 2021). Sec61 binding to the ribosome induces a conformational change that partially opens Sec61's lateral gate without displacing the central plug (Ge et al. 2014; Voorhees et al. 2014). The signal engages the lateral gate with the amino-terminal flanking domain facing the cytosol (i.e., an N_{cyt} topology), causing the carboxy-terminal flanking domain to be pulled through Sec61's central channel (Fig. 3A; Mothes et al. 1994; Li et al. 2016; Voorhees and Hegde 2016). The signal is now at the lipid–Sec61 interface (Martoglio et al. 1995), the plug is displaced, and translocation has been initiated.

Further elongation results in continued translocation until a TMD emerges from the ribosome. Because the polypeptide is threaded through Sec61, this TMD necessarily enters the channel (Do et al. 1996). By this point, the signal peptide is often no longer at the lateral gate, having diffused into the membrane in which it is cleaved by the signal peptidase complex (Lyko et al. 1995; Liaci et al. 2021). The lateral gate dynamically samples open and closed states (Mercier et al. 2021), allowing the nascent protein inside the central channel to periodically sample the membrane. A sufficiently hydrophobic segment of polypeptide can enter the lipid bilayer by passive partitioning (Heinrich et al. 2000; White and von Heijne 2005; Hessa et al. 2007; Öjemalm et al. 2011). For a single-TMD protein, the remainder of the polypeptide passes into the cytosol through a gap between the ribosome and Sec61. Multipass proteins have additional TMDs that emerge from the ribosome, the insertion of which is considered later.

Type II Membrane Proteins

Proteins that are cotranslationally inserted with their amino terminus facing the cytosol are known as type II membrane proteins. The first TMD of these proteins, which will be in the N_{cyt} topology, initiates SRP-mediated targeting and engages the Sec61 complex similar to cleavable signal peptides (Görlich and Rapoport 1993; High et al. 1993a,b; Martoglio et al. 1995), but is not processed by signal peptidase (Fig. 3A). Polypeptide downstream from the TMD is translocated through the Sec61 channel. For a single-spanning type II membrane protein, completion of downstream polypeptide translocation and TMD partitioning into the membrane produces the final protein.

For multipass proteins, the second TMD necessarily begins entering the Sec61 channel when it emerges from the ribosome, then inserts into the membrane in the N_{exo} topology similar to the first TMD of type I proteins. Sequential and independent insertion of the first and second TMD can occur in some cases (Wessels and Spiess 1988; Sadlish et al. 2005). In other cases, TMD2 seems to insert into the membrane together with TMD1 if both of them have partial hydrophilic character (Skach and Lingappa 1993; Lin and Addison 1995; Heinrich and Rapoport 2003; Cymer and von Heijne 2013). Here, partial hydrophilicity of TMD1 would favor its sampling or retention at the lateral gate. TMD2 would interact with TMD1 via their respective low-hydrophobicity regions (Moore et al. 2008; Mravic et al. 2019), thereby favoring membrane partitioning of the two-TMD unit.

Type III Membrane Proteins

Type III membrane proteins begin with a short amino-terminal translocated tail followed by an N_{exo} TMD. Cross-linking experiments with mammalian ER (High et al. 1993a; Heinrich et al. 2000) and reconstitution experiments

with purified Sec61 (Oliver et al. 1995; Heinrich et al. 2000) have long suggested that TMDs of type III proteins insert via Sec61's lateral gate concomitant with N-tail translocation through Sec61's central channel (Matlack et al. 1998; Shao and Hegde 2011). However, type III protein insertion in mammals is not impaired by biochemical or genetic Sec61 depletion (Chitwood et al. 2018; O'Keefe et al. 2021) or multiple Sec61 inhibitors that seem to prevent channel opening (Baron et al. 2016; McKenna et al. 2017; Tranter et al. 2020; O'Keefe et al. 2021). Furthermore, the cryo-electron microscopy (cryo-EM) structure of a type III protein at the point of insertion showed a closed Sec61 lateral gate, with the substrate's TMD possibly residing at a site behind Sec61 (Braunger et al. 2018).

These seemingly conflicting data can be (mostly) resolved by a model in which type III proteins are inserted by an Oxa1 family member (Fig. 3B) followed by docking of the ribosome-nascent chain complex at a SecY family channel. A role for Oxa1 in N-tail translocation during type III membrane protein insertion has long been known for the mitochondrial inner membrane (Hell et al. 1998), with its homologs YidC and Alb3 mediating this reaction in bacteria and chloroplast thylakoid membranes, respectively (Moore et al. 2000; Samuelson et al. 2000). This mechanism is SecY-independent, and can also insert type II membrane proteins with short translocated loops (Fig. 3C; Facey et al. 2007). The finding of Oxa1 family members in archaea (Luirink et al. 2001; Yen et al. 2001; Borowska et al. 2015) and the ER (Anghel et al. 2017) indicated that Sec61-independent insertion might also occur in these membranes.

The ER contains three Oxa1 family members: Get1, EMC3, and TMCO1. Get1 and EMC3 are each central components of larger complexes known to mediate TA membrane protein insertion (Mariappan et al. 2011; Wang et al. 2014; Guna et al. 2018). The nine-protein ER membrane protein complex (EMC) also mediates cotranslational insertion of type III proteins (Chitwood et al. 2018; O'Keefe et al. 2021). Not only has this reaction been reconstituted with purified EMC, but its loss from the mammalian ER impairs insertion of many such substrates. A unifying model that explains these findings posits that an Oxa1 family member transiently samples the nascent chain between the steps of SRP-mediated targeting and ribosome docking onto SecY (Fig. 4). During this sampling step, the Oxa1 family member would insert type III TMDs concomitant with N-tail translocation but would reject signal peptides and type II TMDs, both of which then engage SecY.

The Oxa1 family member in this model would be YidC in *E. coli* and EMC in the ER. Photo-cross-linking experiments show that YidC is indeed adjacent to the SRP–SR complex (Welte et al. 2012; Petriman et al. 2018). Although similar cross-linking has not been reported for EMC, biochemical analysis shows that loss of EMC results in insertion of a type III protein in the N_{cyt} topology, presumably by Sec61 (Chitwood et al. 2018). In these experiments, some substrates hardly required EMC for correct insertion, including the widely studied model TMD from *E. coli* leader peptidase. This might be the result of insertion by Sec61, which was shown to mediate leader peptidase TMD insertion in a purified system (Heinrich et al. 2000). Other possibilities include insertion by another Oxa1 family member (e.g., TMCO1) or unassisted insertion (White and Wimley 1999).

The above model is attractive because the same universal factors (SRP, Oxa1, SecY) participate in the same sequence of events using similar mechanisms in all organisms. Furthermore, this framework explains why insertion of type III proteins is not impaired by Sec61 inhibition or depletion in eukaryotes or SecY depletion in prokaryotes. Because the ribosome eventually docks onto Sec61, a TMD displayed just outside the exit tunnel of a stalled ribosome-nascent chain complex would necessarily cross-link to Sec61α in such end-point assays (High et al. 1993a; Heinrich et al. 2000). This would be the case even if the TMD had been inserted at a prior step by another factor (Chitwood et al. 2018) and is not necessarily at the Sec61 lateral gate (Braunger et al. 2018). Importantly, the model provides a useful framework for how topology is determined, as discussed next.

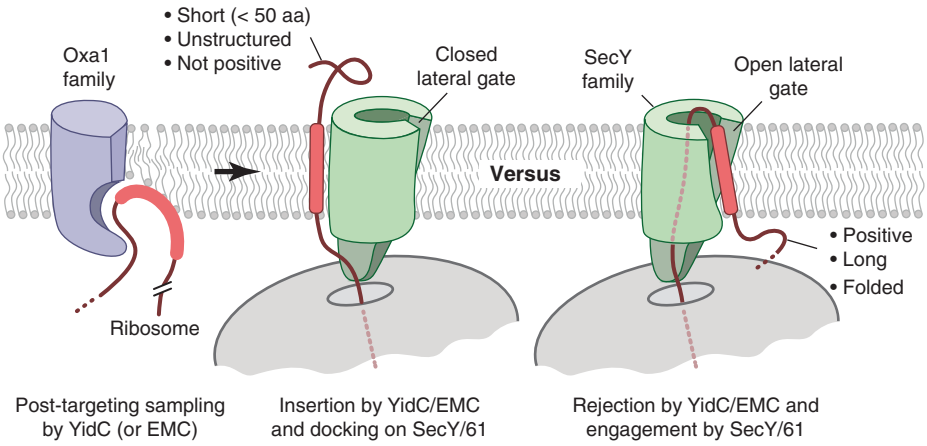

Figure 4. Two-step model for topology determination. Nascent chains target to the membrane using the signal-recognition particle (SRP) system (not depicted) and initially sample an Oxa1 family member (YidC in prokaryotes and endoplasmic reticulum [ER] membrane protein complex [EMC] at the eukaryotic ER). If certain criteria are met by the nascent protein, it is inserted by the Oxa1 family member after which the ribosome docks at the SecY complex (prokaryotes) or Sec61 complex (eukaryotes). Based on cryo-electron microscopy (cryo-EM) of such an intermediate (Braunger et al. 2018) and site-specific photo-cross-linking of the inserted transmembrane domain (TMD) to Sec61γ (Heinrich et al. 2000), the TMD at this stage would be on the hinge side of a closed Sec61 channel. If the substrate does not meet the criteria for insertion by the Oxa1 family member, it is rejected. The ribosome then docks at SecY or Sec61, where the substrate is inserted in the N_{cyt} orientation via the lateral gate. In this model, orientation is therefore determined primarily by the substrate preference of Oxa1 family members. Although not shown, almost all Oxa1 family members have one or more conserved positive charges (typically an arginine) in the hydrophilic vestibule. This might disfavor translocation of TMD-flanking domains enriched in positive charges to enforce the positive inside rule.

DETERMINANTS OF PROTEIN TOPOLOGY

The sequence determinants of protein topology have been extensively studied, using both bioinformatics and mutagenesis of model membrane proteins (Higy et al. 2004; von Heijne 2006). Orientation of the first hydrophobic domain (signal peptide or TMD) typically constrains the orientations of downstream TMDs (with some exceptions [Gafvelin and von Heijne 1994; Hedin et al. 2010; Öjemalm et al. 2012]), which necessarily alternate between N_{exo} and N_{cyt}. The first hydrophobic domain is oriented on the basis of three main parameters: (1) positive charges that flank a TMD (or signal peptide) tend to be retained in the cytosol, an observation known as the positive inside rule (von Heijne 1986; Beltzer et al. 1991); (2) long or folded amino-terminal tails are retained in the cytosol (Denzer et al. 1995); and (3) increased hydrophobicity of a TMD tends to favor the N_{exo} topology when otherwise unconstrained by the first two parameters (Wahlberg and Spiess 1997).

How these features are interpreted by the machinery of protein translocation have long remained murky. Based on the SRP–Oxa1–SecY sequence of events described in the previous section, we propose the following model for topology determination (Fig. 4). After SRP-mediated targeting, an Oxa1 family member would encounter the nascent chain before the ribosome docks onto SecY. Substrates with positively charged or long N-tails would be poor substrates for Oxa1 family members because of repulsion by conserved positive charge(s) inside the hydrophilic vestibule (Kumazaki et al. 2014; Bai et al. 2020; McDowell et al. 2020; Miller-Vedam et al. 2020; O'Donnell et al. 2020; Pleiner et al. 2020). These substrates are effectively "rejected" during the brief window of access to the Oxa1 family member, instead arriving at SecY,

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which inserts the substrate in the N_{cyt} orientation. At this step, positive charges flanking the TMD might be retained in the cytosol through interactions with negative charges on phospholipids or the phosphate backbone of rRNA.

A role for an Oxa1 family member in topology determination would explain why loss of EMC influences topogenesis of an N_{exo} TMD (Chitwood et al. 2018; Chitwood and Hegde 2019) and why mutagenesis of Sec61 surface charges cannot clearly rationalize the positive inside rule (Goder et al. 2004; Junne et al. 2007). Furthermore, a transient window for EMC-mediated insertion prior to reaching Sec61 might explain why high hydrophobicity TMDs, whose insertion is presumably faster and more efficient, favors the N_{exo} topology (Wahlberg and Spiess 1997). Thus, much of the data on topogenesis can be rationalized by a two-step model in which EMC inserts its preferred substrates in the N_{exo} topology, whereas Sec61 inserts EMC-skipped substrates in the N_{cyt} topology. The same model would apply to YidC and SecY in *E. coli*, consistent with an approximately universal set of topologic rules.

THE ITERATIVE INSERTION MODEL FOR MULTIPASS PROTEINS

After the initiation steps described so far (Fig. 3), the ribosome is docked at the Sec61 channel with the most recently inserted TMD (either the first or second) in the N_{exo} topology. Most of the roughly ~30–40 amino acids of polypeptide downstream of this TMD resides in the ribosome exit tunnel. The next two TMDs that will emerge from the ribosome should be successively inserted in the N_{cyt} and N_{exo} orientations. This would occur without another SRP-mediated targeting step because the ribosome is already at Sec61.

In the classic iterative model for multipass protein insertion (Blobel 1980; Matlack et al. 1998), the mechanism of insertion of these two TMDs would follow the same mechanism described above for the first two TMDs of a type II protein (Fig. 5). Thus, the next TMD would emerge from the ribosome, engage the lateral gate of Sec61 in the N_{cyt} topology, and either pass into the membrane or remain at the lateral gate depending on its hydrophobicity. The N_{cyt} orientation is favored by the N-flank being constrained to the cytosol by the preceding TMD. The C-flank is translocated through the Sec61 channel until a subsequent TMD emerges and inserts into the membrane in the N_{exo} topology. This sequence of steps would be iterated until all TMDs have been inserted.

In this model, cytosolic loops are extruded through a gap between Sec61 and the bound ribosome (Pfeffer et al. 2015), whereas lumenal loops are translocated through the Sec61 channel. All TMDs pass through Sec61's lateral gate, with each N_{cyt} TMD responsible for initiating translocation and each N_{exo} TMD responsible for terminating translocation. Interactions between an N_{cyt} TMD and the subsequent N_{exo} TMD would shield their respective hydrophilic regions and permit insertion of low-hydrophobicity TMDs that are unsuitable on their own. Low-hydrophobicity N_{cyt} TMDs would open Sec61 similar to signal peptides, whose hydrophobicity is typically lower than most TMDs (Guna and Hegde 2018).

A CRITICAL ASSESSMENT OF THE ITERATIVE INSERTION MODEL

The central mechanistic feature of the iterative insertion model is that each TMD (other than TMD1 of a type III protein) accesses the lipid bilayer via the lateral gate of Sec61. This is strongly supported for signal peptides and single-TMD type II proteins, and partially supported for single-TMD type III proteins. The requirement for Sec61 was established for signal peptides and a model type II protein in immunodepletion studies (Görlich and Rapoport 1993; Brambillasca et al. 2005; Chitwood et al. 2018) and its sufficiency was shown for model signal peptides, type II proteins, and type III proteins using purified Sec61 (Görlich and Rapoport 1993; Oliver et al. 1995; Hegde et al. 1998; Heinrich et al. 2000). Furthermore, signal peptides and type II proteins are sensitive to several unrelated Sec61 inhibitors that all seem to prevent lateral gate function (Maifeld et al. 2011; Junne et al. 2015; Baron et al. 2016; Paatero et al. 2016; Zong et al. 2019; Tranter et al. 2020). In *E. coli*, SecY with a stapled lateral gate

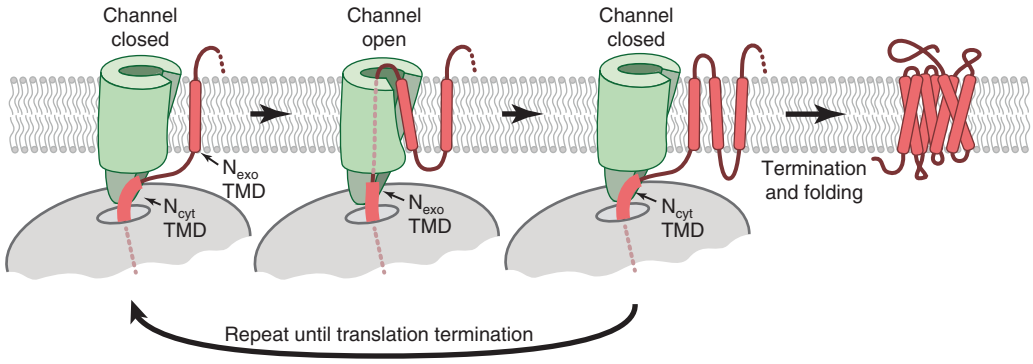

Figure 5. Iterative insertion model for multipass proteins. Model for how transmembrane domains (TMDs) are inserted downstream from a just-inserted N_{exo} TMD (see Fig. 3 for how this initial insertion is achieved). The next TMD emerges from the ribosome and engages the lateral gate of SecY (prokaryotes) or Sec61 (eukaryotes) in the N_{cyt} topology. This orientation is enforced by the N-flank being restricted to the cytosolic side by the preceding TMD. With further elongation, the C-flank translocates through the now open Sec61 channel into the lumen. When the next TMD emerges, it enters the channel and passes into the membrane, sometimes together with the preceding TMD, and the Sec61 channel closes. At this step, the most recently inserted TMD is again in the N_{exo} topology, and the cycle can repeat for additional downstream TMDs until translation terminates.

cannot engage a signal peptide (du Plessis et al. 2009). Finally, N_{cyt} substrates have been visualized at the open lateral gate by crystallography and cryo-EM for both prokaryotic SecY and eukaryotic Sec61 (Li et al. 2016; Voorhees and Hegde 2016).

However, there are exceptions to this Sec61-mediated mechanism when the first translocated domain of a multipass protein is short. For at least one *E. coli* protein (MscL), the first two TMDs and short intervening loop of a type II protein is translocated by YidC after SRP-mediated targeting (Facey et al. 2007). Similarly, the signal peptide and first TMD of the type I multipass protein CyoA also insert in a YidC-dependent and SecY-independent process (Celebi et al. 2006; Van Bloois et al. 2006). Based on how YidC mediates type III protein insertion, the simplest explanation is that two adjacent hydrophobic domains insert as a unit, with YidC facilitating translocation of the short intervening loop via its hydrophilic vestibule (Fig. 3C). It remains to be investigated whether Sec61-independent insertion, perhaps using EMC, occurs for any eukaryotic type II multipass proteins whose first translocated domains are short.

The use of Sec61's lateral gate by subsequent TMDs (hereafter termed late TMDs) has long been assumed but has only modest experimental support. Analysis of at least some late TMDs taken out of context show that they can be targeted and inserted similar to initial TMDs, presumably via the Sec61 lateral gate (Foster et al. 2000; Hedin et al. 2010). However, it remained possible that insertion of late TMDs in their native context differs from how they are handled at the beginning of a protein. In a separate experiment, site-specific photo-cross-linking studies with probes in each TMD of a six-TMD protein showed that they are all near Sec61 when they first emerge from the ribosome (Sadlish et al. 2005). However, because Sec61 is docked at the ribosome exit tunnel, proximity at this stage is to be expected and cannot be used to infer passage through the lateral gate.

There are three reasons to question the idea that late TMDs always insert the same way as initial TMDs as posited by the iterative insertion model. First, sequence analysis shows that late TMDs are less hydrophobic than early TMDs, with many being remarkably hydrophilic (Fig. 6A). Although not extensively analyzed, many late TMDs cannot function to open Sec61 when tested out of their native context (Enquist et al. 2009; Hedin et al. 2010). Second, global analysis of proteins impacted by a Sec61

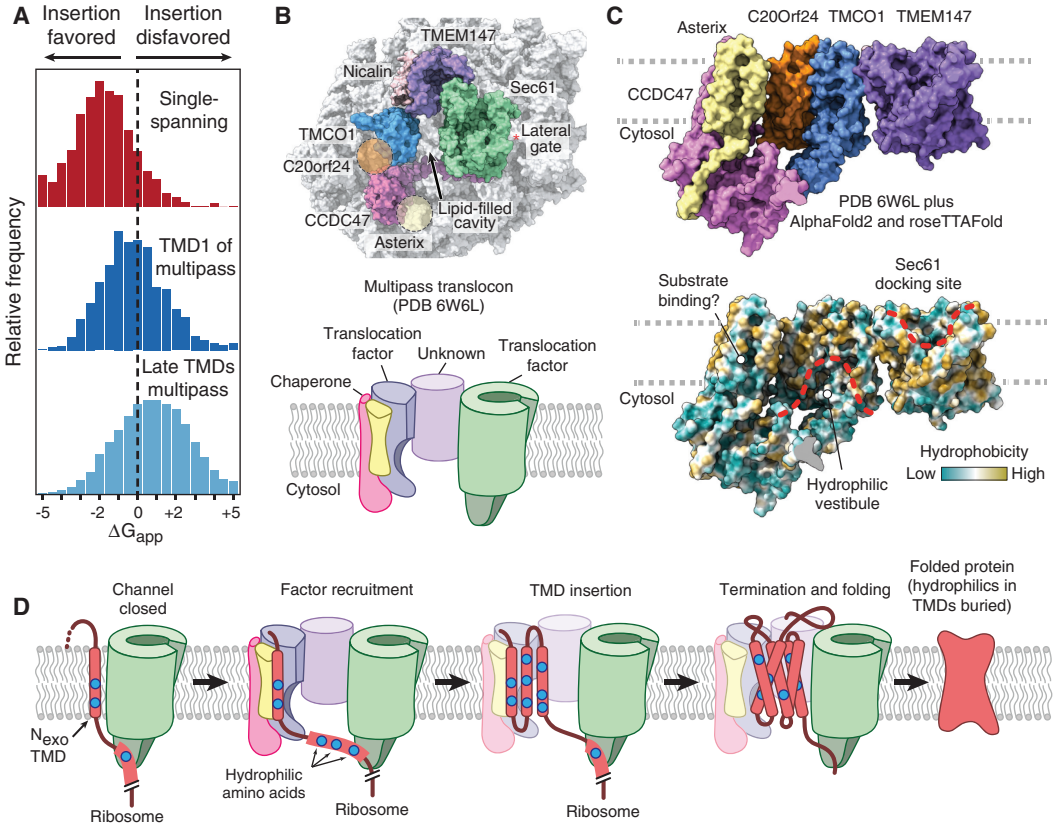

Figure 6. Model for protein insertion by the multipass translocon. (*A*) Histograms of the calculated ΔG_{app} (apparent ΔG) for membrane insertion of the indicated types of transmembrane domains (TMDs) in the human genome (for ~2000 single-TMD proteins and ~2500 multipass proteins). A value of 0 corresponds to 50% insertion, with negative values favoring insertion and positive values disfavoring insertion (Hessa et al. 2007). (*B*) Structure of the multipass translocon viewed from the endoplasmic reticulum (ER) lumen (McGilvray et al. 2020). The predicted positions of the partners for CCDC47 and TMCO1 (Asterix and C20Orf24, respectively) are indicated. The translocon proteins surround a lipid filled cavity. *Bottom* diagram shows the multipass translocon annotated with each factor's proposed role. (*C*) Structural model of the interior of the surface of the multipass translocon that faces the lipid-filled cavity. Predicted structures of the PAT complex (Humphreys et al. 2021) and the TMCO1–C20Orf24 complex (Lewis and Hegde 2021) were docked into their sites based on the positions of CCDC47 and TMCO1 in the multipass translocon structure (PDB 6W6L). The *bottom* shows the hydrophobic surfaces. (*D*) Model for how the multipass translocon might insert a pair of TMDs downstream from a just-inserted N_{exo} TMD (see Fig. 3 for how this initial insertion is achieved). As the downstream TMD emerges, the first TMD engages the PAT complex via its semihydrophilic TMD (Chitwood and Hegde 2020). Other components of the multipass translocon might be recruited at the same time (McGilvray et al. 2020). Once the next TMD also emerges, they insert as a pair using the hydrophilic interior of the multipass translocon for translocation of the intervening loop by a mechanism as in Figure 3C. The lipid-filled cavity is postulated to be a site for membrane protein folding until intramembrane hydrophilic surfaces are buried, triggering release from the PAT complex (Chitwood and Hegde 2020). A multipass protein could use this mechanism and the mechanism shown in Figure 5 for different pairs of TMDs, depending on their properties and intervening flanking domain.

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inhibitor showed that sensitivity was strongly dependent on the first hydrophobic domain, with type III proteins being preferentially refractory to inhibition (Morel et al. 2018). This result is unexpected by the iterative insertion model because later TMDs of a type III multipass protein would need to use the Sec61 lateral gate even if the first TMD used EMC. Third, multipass proteins can be entirely inserted by Oxa1 in mitochondria (Hell et al. 2001; Eaglesfield and Tokatlidis 2021) and by YidC in *E. coli* (Kuhn 1988; Van der Laan et al. 2004; Facey et al. 2007) as long as they do not contain long translocated domains.

It is noteworthy that in membrane proteins with four or more TMDs, the vast majority of translocated loops are shorter than 50 amino acids, averaging ~15 amino acids (Wallin and von Heijne 1998). Indeed, many multipass proteins do not have any large exoplasmic domains, so they can potentially be inserted using solely the Oxa1-like mechanisms shown in Figure 3B,C. A comprehensive genome-wide analysis of translocated loop length with accurate topology information derived from high-quality structure predictions (Tunyasuvunakool et al. 2021) would undoubtedly be informative. In lieu of this, it is noteworthy that among the well-annotated family of 819 human GPCRs, only one of the 106 translocated domains longer than 70 amino acids resides between two TMDs. The rest are located between an amino-terminal signal peptide and TMD1, so are necessarily translocated through Sec61. This selection against long inter-TMD loops seems to be topologically biased because 28 of the 140 cytosolic domains longer than 70 amino acids reside between two TMDs (the rest are at the carboxyl terminus). The iterative insertion model would not predict this curious bias because each loop would translocate through Sec61, and therefore not be constrained by length. In contrast, translocation by Oxa1 family members is length-constrained, hinting at their role during late TMD insertion as discussed later.

CHAPERONES FOR MULTIPASS MEMBRANE PROTEINS

The partial hydrophilicity of TMDs from multipass proteins, even the first one, poses an obstacle to their insertion and stability in the lipid bilayer. This instability is ultimately resolved when the TMDs pack into a folded structure in which the regions facing the lipid bilayer are predominantly hydrophobic (Vinothkumar and Henderson 2010; Cymer et al. 2015; Mravic et al. 2019). Although this problem has been recognized for a long time, chaperones that stabilize membrane protein insertion intermediates are poorly defined.

Oxa1 family members are attractive candidates for membrane protein chaperones (Hennon et al. 2015). They can potentially use their hydrophilic grooves to bind and stabilize semi-hydrophilic TMDs in the membrane. Consistent with this idea, a nascent inserting TMD can be cross-linked to YidC (Scotti et al. 2000; Beck et al. 2001; Chen et al. 2002) and EMC may be near a subset of nascent membrane proteins (Shurtleff et al. 2018). EMC also associates with membrane proteins post-translationally, further suggesting a chaperone function (Satoh et al. 2015; Tang et al. 2017; Shurtleff et al. 2018; Coelho et al. 2019). Studies of the LacY transporter have shown that it does not fold properly without YidC despite having been inserted in the correct topology (Zhu et al. 2013; Serdiuk et al. 2016). Although analogous studies for EMC in membrane protein folding are currently lacking, the diverse pleiotropic phenotypes seen in the absence of EMC are consistent with this idea (Volkmar and Christianson 2020; Hegde 2022).

Another protein sometimes postulated to be a chaperone in eukaryotes is TRAM1. This abundant ER protein is within cross-linking distance of signal peptides and TMDs at the time of their insertion (Görlich et al. 1992; High et al. 1993b; Do et al. 1996; Heinrich et al. 2000). Although an effect on signal peptide function has been documented (Görlich et al. 1992; Voigt et al. 1996), functional consequences of its deletion on TMD insertion or membrane protein folding remain to be shown.

The PAT complex, a heterodimer of the three-TMD protein Asterix and the single-TMD protein CCDC47, has been proposed recently to be a chaperone for multipass proteins (Chitwood and Hegde 2020). Asterix directly interacts with a multipass substrate's TMD dur-

ing, but not after, successful substrate folding. PAT complex depletion partially impairs biogenesis of several unrelated multipass proteins, but not single-TMD proteins. The specificity for multipass proteins may be related to the PAT complex's specificity for semihydrophilic TMDs, which are found in essentially all multipass proteins (Fig. 6A). The combination of direct physical association with TMDs within the membrane and functional consequences for many membrane proteins supports the idea that the PAT complex is an intramembrane chaperone. As described next, it seems to function as part of a larger assembly known as the multipass translocon (Hegde and Keenan 2022).

A TRANSLOCON SPECIALIZED FOR MULTIPASS PROTEINS

The recognition that EMC3, Get1, and TMCO1 are Oxa1 family members (Anghel et al. 2017), together with the now well-established roles in TMD insertion for EMC3 and Get1 (Mariappan et al. 2011; Chitwood et al. 2018; Guna et al. 2018), implicates TMCO1 in membrane biogenesis. This idea has support from biochemical and structural analysis of affinity-purified mammalian TMCO1-associated ribosomes (McGilvray et al. 2020). Sequencing of the mRNAs associated with these ribosomes found a strong enrichment for multipass membrane proteins, implicating TMCO1 in their biogenesis. Furthermore, mass spectrometry showed that these ribosomes also contain CCDC47 and a previously defined complex of TMEM147, Nicalin, and NOMO (Dettmer et al. 2010). Although Asterix (the obligate partner of CCDC47) and C20Orf24 (the predicted partner of TMCO1) were not reported, this might have been because of a paucity of tryptic peptides for mass spectrometry. Strikingly, each of these components were strong hits in a genome-wide CRISPR screen for surface expression of the multipass TRPC6 channel (Talbot et al. 2019).

Structural analysis of TMCO1-associated ribosomes revealed its position, along with those of TMEM147, Nicalin, and CCDC47, relative to the Sec61 channel (Fig. 6B; McGilvray et al. 2020). The moderate resolution structure did not reveal NOMO, Asterix, or C20Orf24. Nonetheless, the latter two can be inferred based on high-confidence predictions of their complexes with CCDC47 and TMCO1, respectively (Fig. 6C; Humphreys et al. 2021; Lewis and Hegde 2021; Mirdita et al. 2021). Together with the functional data on the PAT complex (Chitwood and Hegde 2020), what emerges is a translocon that may be specialized for multipass proteins. Notably, these new components were not observed on ribosomes producing secretory proteins, in which oligosaccharyl transferase (OST) is seen instead at this site (Pfeffer et al. 2015; Braunger et al. 2018). This implies that the multipass translocon is assembled in response to a multipass protein substrate. Based on the timing of PAT complex interaction after TMD1 insertion of a type III protein but before TMD2 insertion (Chitwood and Hegde 2020), the assembly would correspond to the transition between early versus late TMDs insertion of a multipass protein. This observation implicates a role for the multipass translocon in the insertion or folding of late TMDs of multipass proteins.

MODEL FOR MULTIPASS PROTEIN BIOGENESIS

Drawing on the various findings from different experimental systems discussed to this point, we argue that the iterative insertion model provides an incomplete explanation for how many (or perhaps most) native multipass proteins are inserted. Instead, an updated framework that combines features of the iterative model with roles for the Oxa1 family and intramembrane chaperones is proposed. The framework should be considered speculative, but we have tried to make it specific, consistent with most of the findings, and testable.

After the initiation steps of multipass proteins (Fig. 3), the inserted TMD(s) would reside at the back (or hinge) of Sec61, not at the frontside lateral gate (Fig. 6D). This idea is based on two sets of findings. First, the cryo-EM structure of a type III protein immediately after insertion showed an unoccupied lateral gate and an unassigned TMD between the back of Sec61 and OST (Braunger et al. 2018). A reconstruction lacking substrate did not have this TMD density, suggesting it

might be the substrate. This position would explain why a photo-cross-linking probe in the center of TMD1 for a model type III protein cross-links to Sec61γ (Heinrich et al. 2000), whose entire TMD resides on the backside (Voorhees et al. 2014). Residence of an N_{exo} TMD at the hinge, even for those inserted via Sec61, might occur because tension in the nascent chain favors the position nearest the ribosome exit tunnel.

The second argument for (re)location of the initially inserted N_{exo} TMD to the hinge side is the subsequent substrate interaction with the PAT complex located on this side (Fig. 6D). Although the timing or mechanism of OST replacement by the multipass translocon is unknown, it presumably occurs before the second or later lumenal loops have been translocated. This is because only the first loop tends to be glycosylated cotranslationally, consistent with OST leaving the translocon at later stages (Cherepanova et al. 2019). Once an early TMD engages the PAT complex at its site behind Sec61, subsequent polypeptide would be directed away from the lateral gate and into the space between the ribosome and membrane on the hinge side of Sec61. Redirection of the nascent chain might be further aided by CCDC47, whose carboxy-terminal domain is near the ribosome exit tunnel and partially occludes access to the central channel of the Sec61 complex (McGilvray et al. 2020).

In the context of the mammalian multipass translocon, this peninsula of lipid behind Sec61 is surrounded on three sides by membrane proteins (Fig. 6B). Notably, the surfaces of Asterix, TMCO1, and C20Orf24 that would face this lipid cavity are substantially hydrophilic and conserved (Fig. 6C). The membrane might therefore be distorted or thinned in this region, reducing the barrier to translocation (Wu and Rapoport 2021). In organisms that lack TMCO1, another Oxa1 family member such as EMC could operate in its place. Consistent with this idea, the EMC3-EMC6 core can partially replace the function of mitochondrial Oxa1 (Güngör et al. 2022) and proximity-based ribosome profiling in yeast shows EMC near some nascent multipass proteins (Shurtleff et al. 2018). We therefore posit that two closely spaced TMDs emerging from the ribosome in the proximity of TMCO1 (or EMC) could insert as a pair into this backside region (Fig. 6D). The translocated loops between most late TMDs are short and amenable to an Oxa1-type insertion mechanism.

The fact that this backside region is lined by multiple hydrophilic membrane proteins (Fig. 6C) means even relatively hydrophilic pairs of TMDs could be stabilized in the membrane. The region between Sec61 and the multipass translocon components is large enough to house around six or seven substrate TMDs (Fig. 6D). Most membrane proteins with more than seven TMDs are modular, having evolved by fusion of smaller multipass proteins (von Heijne 2006). Thus, the cavity behind Sec61 would be able to accommodate the basic units that comprise most multipass membrane proteins. Accumulation of multiple TMDs in this region during biogenesis would explain why insertion intermediates with up to six TMDs remain extractable with agents such as urea or alkali (Borel and Simon 1996). The semiprotected cavity behind Sec61 might also provide an environment amenable to rearrangement of protein topology (Lu et al. 2000).

In the (relatively rare) instances where an N_{cyt} TMD is followed by a long translocated domain (Wallin and von Heijne 1998), pairwise TMD insertion on the backside by an Oxa1 family member would be disfavored. In this situation, the N_{cyt} TMD would engage the Sec61 lateral gate (Fig. 5D), and the subsequent long loop would be translocated through Sec61 until the next TMD emerges and inserts in the N_{exo} topology. For Sec61 to be accessed, the multipass translocon (and in particular, CCDC47) might need to be displaced. How this occurs remains to be determined but could be triggered by the accumulation of non-translocated polypeptide in the constrained space between the ribosome and translocon. Long translocated loops might also impose certain hydrophobicity constraints on the flanking TMDs to ensure their capacity to use Sec61. Indeed, the sole long internal loop in human GPCRs (found in C3AR1) is flanked by two TMDs that are far more hydrophobic that what is normally seen there in other GPCRs. Thus, multipass membrane proteins would use Sec61 for insertion of those TMDs separated by a long translocated domain and the multipass

translocon for TMDs with short translocated loops (i.e., a combination of what is shown in Figs. 5 and 6D).

For multipass proteins with only short translocated domains, insertion can occur without using SecY at all, as demonstrated for several "YidC-only" proteins in *E. coli* and the entire complement of mitochondrially encoded membrane proteins inserted by Oxa1. In mammals, the sequential use of EMC for the first TMD and the multipass components for the remainder could explain why type III multipass proteins seem to be impervious to Sec61 lateral gate inhibition. Yet, insertion would occur almost entirely adjacent to Sec61, explaining earlier data showing that each TMD cross-links to Sec61 shortly after its insertion and sometimes remains in Sec61's proximity for a prolonged period (Sadlish et al. 2005).

In eukaryotes, and especially metazoans, the task of inserting and chaperoning membrane proteins seems to have become distributed among multiple Oxa1 family members and eukaryote-specific factors such as the PAT complex. In prokaryotes, it is possible that their simpler membrane proteome can be accommodated by a single Oxa1 family member for both insertion and chaperoning. Consistent with this idea, individual Oxa1 family members are each less abundant than Sec61 in eukaryotes (Kulak et al. 2014; Itzhak et al. 2016; Cho et al. 2022), whereas YidC is more abundant than SecY in *E. coli* (Schmidt et al. 2016). Notably, a lipid-filled cavity formed partially of SecY and YidC was proposed to be a site for membrane protein insertion in the *E. coli* system, albeit with a different organization than what is emerging in the mammalian system (Botte et al. 2016). Notwithstanding these differences, the core principle is conserved: SecY and Oxa1 family members collaborate to mediate insertion of different parts of a multipass membrane protein depending on features of the TMDs and their translocated flanking domains.

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