Tail-anchored membrane protein insertion into the endoplasmic reticulum

Ramanujan S. Hegde and Robert J. Keenan‡*

Abstract | Membrane proteins are inserted into the endoplasmic reticulum (ER) by two highly conserved parallel pathways. The well-studied co-translational pathway uses signal recognition particle (SRP) and its receptor for targeting and the SEC61 translocon for membrane integration. A recently discovered post-translational pathway uses an entirely different set of factors involving transmembrane domain (TMD)-selective cytosolic chaperones and an accompanying receptor at the ER. Elucidation of the structural and mechanistic basis of this post-translational membrane protein insertion pathway highlights general principles shared between the two pathways and key distinctions unique to each.

Chaperones
A large group of proteins that facilitate the folding, assembly, transport and degradation of non-native polypeptides by minimizing inappropriate interactions.

**Medical Research Council (MRC) Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 0QH, UK.*
‡Department of Biochemistry and Molecular Biology, The University of Chicago, Gordon Center for Integrative Science, Room W238, Chicago, Illinois 60637, USA.
e-mails: rhegde@mrc-lmb.cam.ac.uk; bkeenan@uchicago.edu
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All biological membranes contain a structurally diverse assortment of integral membrane proteins, which collectively constitute ~30% of the cellular proteome[1,2]. These proteins impart essential functionality to the lipid bilayer to allow a range of cellular activities, including transmembrane communication, transport and membrane morphogenesis. The selective and asymmetric insertion of membrane proteins is therefore an evolutionarily ancient problem that was solved by the earliest life forms.

The shared feature of all integral membrane proteins is the highly hydrophobic transmembrane domain (TMD), which in the final structure resides within the lipid bilayer[3]. Thus, a critical obstacle in membrane protein insertion is the movement of these TMDs from the aqueous cytosol, where they are synthesized, into the lipid bilayer, where they are energetically most stable[4]. This process necessitates selective TMD recognition, shielding of the TMD from the aqueous cytosol, targeting to the membrane surface and integration of the TMD into the lipid bilayer in the correct orientation. All membrane protein insertion pathways must solve these four problems, each of which typically involves specialized and highly regulated factors in the cytosol and target membrane.

In eukaryotes, membrane proteins synthesized on cytosolic ribosomes can be targeted to mitochondria[5], peroxisomes[6], chloroplasts (in plants)[7] and the endoplasmic reticulum (ER)[8]. Among these, the ER accommodates the largest number of proteins, encompassing all membrane proteins of the plasma membrane, compartments of the secretory and endocytic pathways, and both nuclear envelope membranes[9].

Insertion into the ER membrane can occur either co-translationally or post-translationally, each of which offers distinct advantages[10,11]. In the co-translational pathway, all of the steps from initial protein recognition to final insertion into the membrane occur during protein synthesis. By contrast, targeting and insertion via post-translational pathways occur after complete synthesis of the membrane protein substrate. Thus, the ribosome is a major functional component during all steps of co-translational insertion, whereas its role in post-translational pathways is limited to the very earliest steps.

Although the co-translational pathway was discovered over 30 years ago[12–14] and has been extensively studied in many systems[15–19], the post-translational insertion pathway has only recently come into focus. Similarly to the post-translational translocation of soluble proteins into various organelles[20–24], the basic paradigm of post-translational ER membrane protein insertion involves cytosolic chaperones (mediating recognition and shielding) that interact with a specific ER-localized receptor (mediating targeting and insertion). Here, we review this post-translational pathway and discuss how the problems of recognition, shielding, targeting and insertion are solved by its machinery. As we outline, there is now sufficient information about each step to provide a plausible mechanistic framework for the whole pathway. These insights are starting to reveal common themes that are likely to apply to all membrane protein insertion processes.

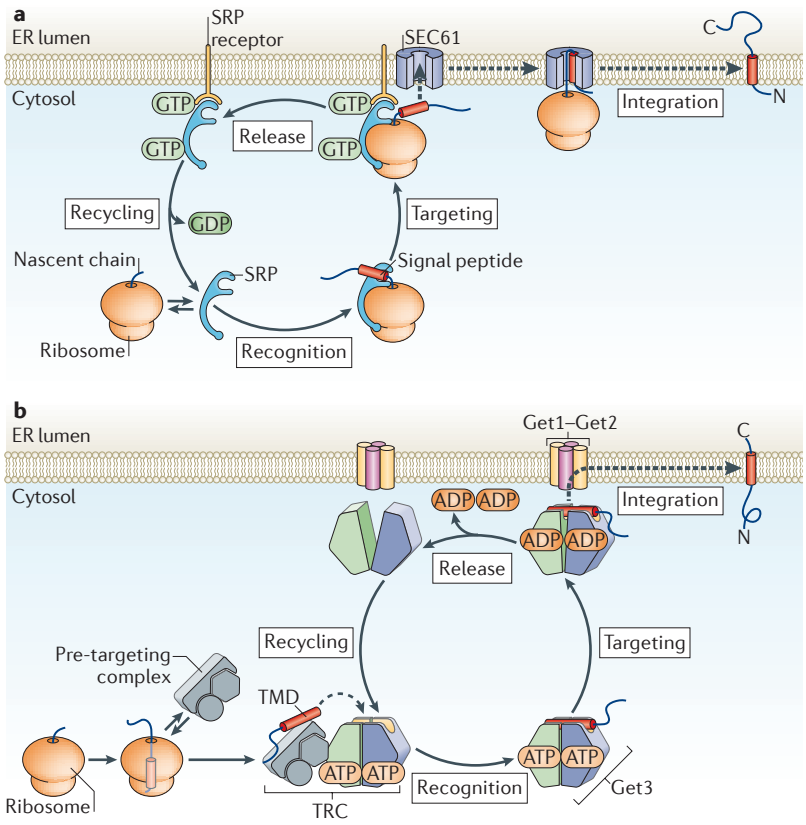

Figure 1 | **Membrane protein biosynthesis in eukaryotes. a** | In the co-translational pathway for the insertion of endoplasmic reticulum (ER) membrane proteins, signal recognition particle (SRP) recognizes the hydrophobic signal peptide of the nascent chain as it emerges from a translating ribosome. The ribosome–nascent chain–SRP complex is targeted to the membrane by a GTP-dependent interaction with the SRP receptor, resulting in the release of the signal peptide and docking of the ribosome–nascent chain complex to the SEC61 translocon. Translation then resumes, and the nascent polypeptide is inserted into the membrane bilayer. After GTP hydrolysis, SRP is recycled to the cytosol. **b** | In the post-translational pathway for the insertion of tail-anchored (TA) ER membrane proteins, a soluble pre-targeting complex captures the hydrophobic transmembrane domain (TMD) of the TA substrate after it emerges from the ribosomal exit tunnel. After loading onto Get3 (TRC40 in mammals), the TA substrate is targeted to the ER membrane by interaction with the Get1–Get2 receptor complex. After ATP has been hydrolysed, the TA substrate is released for insertion into the bilayer. ATP binding recycles Get3 (or TRC40) back to the cytosol. N, amino terminus.

The co-translational pathway

The extensively studied co-translational pathway provides an important conceptual context for understanding post-translational membrane protein insertion. It is therefore worth first summarizing the general features of co-translational membrane protein insertion[8,11,25,26]. This pathway begins when a hydrophobic segment of the protein, typically the first TMD, emerges from the ribosome (FIG. 1). This hydrophobic domain is recognized by signal recognition particle (SRP)[27], a large ribonucleoprotein complex that is composed of multiple proteins and an RNA scaffold[28]. SRP has a high affinity for ribosomes and binds these, through its 54 kDa subunit (SRP54), near the ribosomal exit tunnel[29]. The Met-rich domain (M-domain) of SRP54, which directly binds to hydrophobic domains[30,31], is therefore precisely poised to capture the nascent membrane protein.

Ribosomal exit tunnel
An internal channel in the large subunit of the ribosome through which the nascent polypeptide travels before emerging into the cytosol. Various factors bound to the ribosome surface can affect the folding and/or targeting of the nascent polypeptide as it emerges from the exit tunnel.

After this initial recognition step, the SRP-bound ribosome–nascent chain complex is targeted to the SRP receptor[32,33] at the ER membrane. The ribosome–nascent chain complex is then released from SRP and transferred to the SEC61 complex, the central component of a protein translocon in the ER[16,34,35]. The ribosome subsequently completes the synthesis of the membrane protein while remaining bound to the SEC61 translocon. The SEC61 complex is therefore positioned to recognize each TMD as it emerges from the ribosome and facilitate their integration into the lipid bilayer[36–38].

A major advantage of the co-translational strategy is that the machinery for targeting and insertion is physically coupled to the ribosome near the polypeptide exit tunnel. This spatial organization allows TMDs to be recognized, shielded and inserted with minimal exposure to the bulk cytosol. The co-translational insertion machinery therefore enjoys a considerable competitive advantage in binding TMDs over many of the other potential binding partners in the cell.

More importantly, especially for multi-spanning membrane proteins, the handling of TMDs as they emerge from the ribosome substantially obviates a need to maintain the solubility of highly hydrophobic, lengthy and complicated proteins. Because the machinery for TMD insertion is the same as that which mediates the translocation of soluble proteins, membrane proteins with large soluble domains that undergo translocation do so by the co-translational pathway. Thus, a near-universal theme is that membrane proteins with multiple TMDs or large translocated domains use the co-translational mode of translocation. These proteins constitute the majority of membrane proteins in the cell, and co-translational membrane protein insertion has been described for the ER, the topologically equivalent bacterial plasma membrane[39], the mitochondrial inner membrane[40] and the thylakoids of chloroplasts[41]. The SEC61 complex also mediates the post-translational translocation of soluble proteins in bacteria and eukaryotes[10,11]; as this pathway is not known to mediate membrane protein insertion, it is not considered in this Review.

Discovery of a new insertion pathway

As the SRP-dependent pathway was being elucidated, it became clear that at least some membrane proteins might not be able to use this route for insertion. In particular, the apparently obligate recognition of membrane proteins by SRP during synthesis was noted to be incompatible with proteins that have a single TMD near the carboxyl terminus[42]. This is because the TMD would be inside the ribosomal tunnel (which houses ~40 amino acids of the nascent polypeptide) when the termination codon was reached. This means that TMD recognition would be required to occur after the termination of translation (that is, post-translationally).

Among the first of these tail-anchored (TA) proteins to be examined for its SRP-dependence was synaptobrevin — a SNARE protein that has key roles in intracellular vesicular trafficking. It was rigorously demonstrated that, as predicted, synaptobrevin uses an SRP- and SEC61-independent post-translational pathway for its

Box 1 | Other routes into the ER membrane

In the absence of the Get–transmembrane domain-recognition complex (TRC) system, many tail-anchored (TA) proteins are still able to insert into a membrane *in vitro* and *in vivo* with at least some efficiency. This raises the possibility of additional pathways for TA protein insertion into the endoplasmic reticulum (ER), including an unassisted pathway[44,50,51], a chaperone-mediated pathway involving heat shock protein 70 (HSP70)[52], and a pathway using signal recognition particle (SRP) in a post-translational mode[53]. The mechanisms of these possible routes are not considered here in detail. Nevertheless, it is worth considering whether these are bona fide insertion pathways that are normally operational *in vivo*.

Several observations suggest that these may be ad hoc insertion mechanisms that are only detectable under specialized conditions. First, deletion of Get components in *Saccharomyces cerevisiae* produces TA proteins that are substantially aggregated and/or mislocalized[65–68]. Thus, although enough essential TA proteins do manage to insert into the ER to maintain viability, target specificity and insertion efficiency are compromised for all substrates that have been examined. Second, essentially all of the evidence for these pathways derives from *in vitro* analyses that use one membrane, so targeting specificity is not assayed. Although these systems are powerful, the interpretation of *in vitro* studies merits some caution in the absence of further corroboration. Additionally, the use of crude translation lysates and microsomes poses a substantial problem for interpretation because it is now clear that they contain the Get–TRC-targeting machinery[55,66]. Even if cytosol is replaced by purified factors[52], the addition of microsomes contributes substantial amounts of the Get–TRC machinery (including Get3; TRC40 in mammals). Thus, any factors that can temporarily prevent TA proteins from aggregating may seem to be necessary simply by facilitating capture by the Get–TRC pathway. And finally, *in vitro* crosslinking assays (the primary means of detecting potential targeting factors) can lead to numerous minor 'off-pathway' interactions, given the rather hydrophobic TA substrate and lengthy reaction times. Thus, although it is possible that other specific pathways exist, the rigorous validation and demonstration of their physiological importance await additional studies.

insertion into the ER[43]. Although this and subsequent studies showed that insertion was protein- and ATP-dependent[43–46], the molecular basis of this energy requirement and the factors involved in insertion remained obscure for over 10 years.

During this intervening period, TA proteins were increasingly appreciated to be of broad physiological importance. Representing ~3–5% of all membrane proteins[47–49], TA proteins are found in all cellular membranes and have functions that range from membrane biogenesis to apoptosis, vesicular trafficking, protein degradation and many others. With increased interest in this class of proteins, greater attention was paid to their mechanism of insertion. However, studies of different TA proteins by different methods led to diverse conclusions. Proposals included one of an unassisted mechanism not requiring any insertion machinery (for cytochrome b_5 studied *in vitro*)[44,50,51], one of a heat shock protein 70 (HSP70)-mediated pathway[52], and one in which SRP and SEC61 are used in a post-translational mode[53,54]. The physiological relevance of these potential routes to the membrane remains largely unclear at present (BOX 1).

Eventually, biochemical analysis of TA protein insertion in cell-free translation extracts, combined with protein crosslinking approaches, led to the identification of a factor that associated with the TMDs of TA proteins[55,56] (FIG. 1). This factor, originally annotated Asna1 (REF. 57) (for its similarity to ArsA, an arsenite-transporting ATPase in bacterial systems[58]), was renamed TMD-recognition complex protein of 40 kDa (TRC40). Evidence for its role in TA insertion came from several *in vitro* observations[55,56], each of which was eventually explained by subsequent mechanistic studies.

First, TRC40 associated with the TMDs of TA proteins but not membrane proteins with internal TMDs[55,56]. Second, it was an ATPase (which reconciled the ATP-dependence of TA protein membrane insertion). Third, it was highly conserved across all eukaryotes, was essential in mammals[59], and mutant phenotypes in other organisms were related to processes that involved TA proteins[60–62]. Fourth, an ATPase-deficient mutant acted as a dominant-negative, selectively binding to but not releasing TA proteins, thereby precluding their insertion into the membrane[55]. Fifth, a fraction of TRC40 was found on the ER membrane, and this could be released by ATP[55]. And finally, it contained a proteinaceous binding site (or sites) on ER microsomes[55]. Based on these observations, TRC40 was proposed to be a targeting factor that selectively recognizes TA proteins in the cytosol and delivers them to the ER for insertion in an ATP-dependent manner[55].

The high level of conservation readily identified Get3 (guided entry of TA proteins 3; originally known as Arr4 (REF. 63), again owing to its similarity to ArsA) as the budding yeast homologue of TRC40. Synthetic genetic and physical interaction studies had already defined *GET3* as part of a pathway involving at least two other genes (termed *GET1* and *GET2*), the loss of which led to phenotypes that were consistent with a role in Golgi–ER trafficking (hence the original delineation with the acronym Get)[60,61,64]. However, the physical and functional links of the mammalian homologue, TRC40, to TA protein insertion into membranes, combined with the fact that Golgi–ER trafficking depends on TA proteins, led to a reassessment of the Get pathway. Parsimoniously, all of the otherwise unconnected yeast phenotypes associated with the Get pathway were reconciled as secondary to defects in TA insertion, meriting a change to the current 'guided entry of TA proteins' moniker for the *GET* genes[65]. Subsequent physical and genetic interaction analysis of this Get pathway in yeast led to the identification of three additional factors (termed Get4, Get5 and small Glu-rich tetratricopeptide repeat-containing 2 (Sgt2))[66–68]. This defined the major players in a single experimental system of budding yeast and placed them into either early (cytosolic for Get3, Get4, Get5 and Sgt2) or late (membrane for Get1, Get2 and Get3) steps that made genetic sense. Subsequent insights into the mechanistic roles of these factors have come from a combination of structural and functional studies, primarily of the yeast Get pathway.

Substrate recognition by Get3

Critical to TA protein targeting is its selective and efficient recognition by Get3. The sensitivity of this interaction to detergent and its dependence on the presence of a functional TMD strongly suggested a direct recognition of the TMD via hydrophobic interactions with Get3 (REF. 55). Such an interaction could also shield and maintain the solubility of the hydrophobic TMD as it transits through the cytosol. Insight into both TA substrate recognition and shielding came from structural studies.

Translocon
A membrane channel that is associated with the transport of polypeptides into or across cellular membranes.

SNARE
(Soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein (SNAP) receptor). A family of tail-anchored coiled-coil proteins that regulate fusion reactions and target specificity in vesicle trafficking.

Heat shock protein 70
(HSP70). A ubiquitous family of ~70 kDa heat-shock proteins that serve as molecular chaperones to regulate polypeptide folding, translocation and degradation.

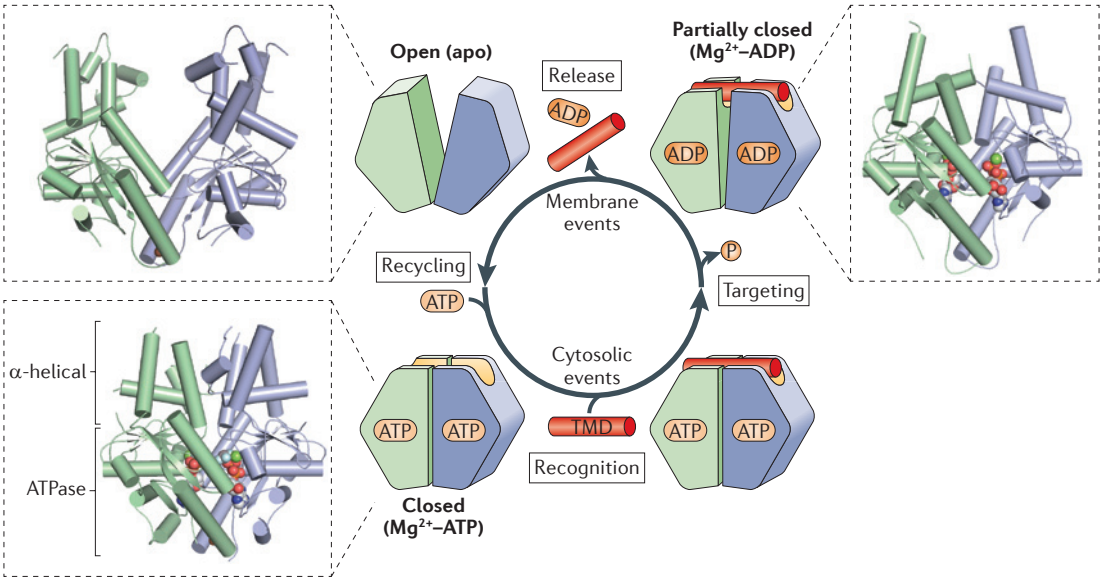

Figure 2 | **Nucleotide-dependent conformational changes in the Get3 homodimer.** Each Get3 monomer comprises two distinct regions: an α-helical subdomain and an ATPase subdomain. In the presence of ATP, the Get3 helical subdomains become intimately associated, forming an extended composite hydrophobic groove (FIG. 3) that recognizes and binds to the transmembrane domain (TMD) of a tail-anchored (TA) substrate. ATP hydrolysis, which occurs at some stage before the release of the TA substrate at the endoplasmic reticulum (ER) membrane, produces an ADP-bound Get3 homodimer that is partially closed. Following the release of the TA substrate and ADP, Get3 shifts back to an open conformation. Subsequently, ATP binding allows recycling of Get3 back to the cytosol in a closed conformation. The insets show crystal structures of the fungal Get3 homodimer in the nucleotide-free state (Protein Databank (PDB) ID 2WOO), the Mg^{2+}–ADP-bound state (PDB ID 3IQX) and the Mg^{2+}–ADP–AlF_4^--bound state (which seems to mimic the ATP-bound state; PDB ID 2WOJ).

Near-simultaneous reports of Get3 crystal structures from multiple fungal species and in multiple states revealed that Get3 was a symmetric homodimer[69–73]. Each Get3 monomer comprises a core ATPase domain decorated with an α-helical domain. The arrangement of Get3 subunits depends on the nucleotide state, transitioning from a fully open state in the absence of nucleotide to a fully closed conformation in the presence of Mg^{2+}–ADP–AlF_4^- (which seems to mimic the ATP-bound state) and a partially closed state in the presence of Mg^{2+}–ADP (FIG. 2). In contrast to the relatively rigid conformation of the ATPase domain, the conformation of the α-helical domain is sensitive to nucleotide binding. In the fully closed, ATP-bound state, the helical subdomains are in direct contact and define a large, hydrophobic groove that spans both Get3 monomers[71] (FIGS 2,3).

Three lines of evidence illustrated that the helical domains mediate recognition. First, the size, shape, hydrophobicity, flexibility and the ATP-dependent formation of the composite groove argued for this being the site of TMD binding[69–72]. Second, hydrogen exchange mass spectrometry (HX-MS) studies demonstrated protection of the α-helical subdomains upon binding to a TA substrate[69]. Third, perturbing the composite hydrophobic groove by introducing negative charges or disrupting dimerization reduced TA substrate binding *in vitro* and led to growth defects *in vivo*[71].

The process of substrate recognition in the co- and post-translational pathways shows important functional and mechanistic similarities. As with Get3, the recognition element in Ffh (the bacterial SRP54 homologue) is constructed from an α-helical protein scaffold that presents a large hydrophobic surface area for substrate binding[74] (FIG. 3). Moreover, these scaffolds are highly dynamic. As two crystal structures of the SRP54–signal peptide complexes show, this flexibility can be leveraged to accommodate targeting signals of different lengths and sequence[75,76]. A similar mechanism is probably at play in the case of Get3, although this awaits further structural analysis of Get3–TMD complexes.

In addition to flexibility in accommodating different sequences, substrate recognition by Get3 must also be selective in at least two ways. First, the C-terminal TMDs of TA proteins must be distinguished from the internal TMDs of co-translational substrates. Second, Get3 must avoid the TMDs of TA proteins destined for other organelles (such as peroxisomes, mitochondria and chloroplasts). As there is little difference between the TMDs of these different substrates[77], a key issue is how the Get pathway selects only the correct substrates for targeting.

One clue comes from the observation that, in the absence of SRP (or other competing factors), Get3 can bind substrates containing internal TMDs. Conversely, SRP cannot recognize a TMD it normally binds when that same TMD is near the C terminus. This suggests that, under physiological conditions, SRP binding to internal TMDs is strongly favoured by its association with a translating ribosome. Hence, its very high local concentration near the ribosomal exit tunnel ensures that SRP will out-compete any other available binding proteins, such as Get3.

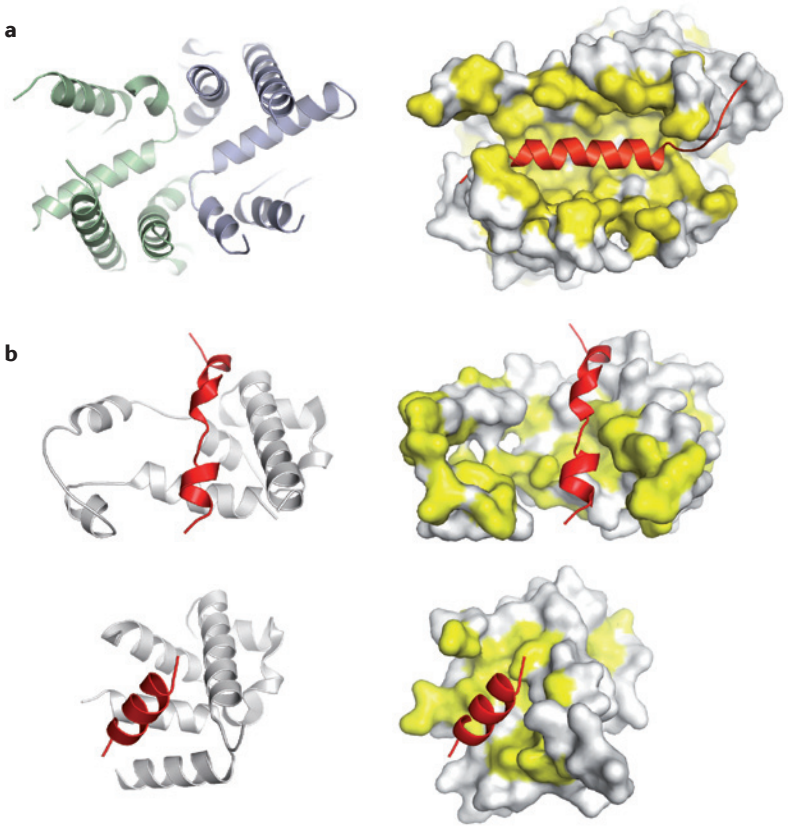

Figure 3 | **Substrate recognition by the post- and co-translational targeting machinery. a** | A large groove in Get3 is formed by the association of the two α-helical subdomains (left panel, green and blue) in the ATP-bound, closed dimer conformation. This composite groove presents a large hydrophobic surface (yellow) for binding to the transmembrane domain (TMD) of a tail-anchored (TA) protein substrate. The TA protein SEC61β (shown in red in the right panel; Protein Databank (PDB) ID 1RHZ) is modelled inside the groove. **b** | Crystal structures of the Met-rich domain (M-domain) of signal recognition particle 54 kDa (SRP54) bound to a signal peptide (shown in red) (top panel, PDB ID 3KL4; bottom panel, PDB ID 3NBD). Note the different, but overlapping, peptide binding sites. For both the post-translational TA protein pathway (**a**) and the co-translational pathway (**b**), the dynamic properties of these helical, hydrophobic scaffolds probably allow them to accommodate different sequences during targeting.

Distinguishing ER-destined TA proteins from other TA proteins is a more difficult problem because they cannot be discriminated on the basis of topological constraints. *In vitro* crosslinking studies in mammalian translation extracts show a clear dependence on hydrophobicity for TRC40 association, with even modest decreases abolishing the interaction[78]. But it is difficult to envision how the flexible hydrophobic groove of Get3 or TRC40 could, by itself, provide tight discrimination between closely related TA substrates. Instead, as described below, additional cofactors acting in conjunction with Get3 or TRC40 probably enhance the fidelity of substrate recognition.

Cofactors for substrate loading

Although the structure of Get3 explained how it could bind and shield the TMD of a TA protein, it was unclear how the TA protein could get loaded onto Get3 in the first place. At least three issues were especially problematic. First, Get3 did not appear to bind ribosomes, raising the problem of how it could capture TA proteins after their release from the ribosome in a sufficiently timely manner to avoid inappropriate interactions and aggregation. Second, it seemed unlikely that the conformation of Get3 that binds TA proteins, which exposes a large hydrophobic surface, would be favoured or long-lived in the aqueous cytosol. Third, it was unclear how Get3 could outcompete other chaperones in the cytosol that also bind hydrophobic domains. Thus, a gap existed in our knowledge between TA protein release from the ribosome and subsequent recognition by Get3.

Genetic analysis of budding yeast had suggested that cytosolic cofactors Get4, Get5 and Sgt2 affected this process, although it was unclear how[66,67]. Insight into this problem came from parallel biochemical studies in the yeast and mammalian systems that converged in supporting a conserved role for pre-targeting cofactors in aiding substrate capture by Get3 or TRC40 (FIG. 4). In yeast, Sgt2 was observed to bind directly to the TMDs of TA proteins, and this interaction was critical for their loading onto Get3 (REF. 79). Importantly, transfer of TA proteins from Sgt2 to Get3 required the Get4–Get5 subcomplex. Interaction analysis further showed that Get4–Get5 forms a scaffold that bridges Sgt2 (which binds to Get5) and Get3 (which binds to Get4). Importantly, Get4 may favour binding selectively to the ATP-bound (and hence closed) conformation of Get3 (REF. 80). This means that Get3 is recruited to Get4–Get5 selectively in a conformation that exposes its hydrophobic TMD binding groove. Thus, an attractive model is that substrates are transferred from Sgt2 to Get3 through the ability of Get4–Get5 to selectively recruit the correct conformation of Get3 in proximity to substrate-bound Sgt2. Precisely how this intricate handover occurs remains to be investigated.

A similar process seems to operate in the mammalian system[78]. In this case, an assay for substrate capture by TRC40 was used to illustrate the need for other factors. Purification of a requisite factor revealed a three-protein complex composed of BAG6 (also known as BAT3 and Scythe), TRC35 and UBL4A. TRC35 and UBL4A are homologous to Get4 and Get5, respectively, and BAG6 can interact with TA proteins, similarly to Sgt2 (although no primary sequence homology is apparent). Depletion of the BAG6 complex resulted in defective TA protein capture by TRC40 (REF. 78) and reduced membrane protein insertion efficiency[81]. Thus, by homology and by functional analysis, pre-targeting cofactors facilitate substrate loading onto Get3 and TRC40 in the yeast and mammalian systems, respectively.

Although mammalian BAG6 and yeast Sgt2 are not related, it is noteworthy that BAG6 interacts with SGTA[82], the mammalian homologue of Sgt2. This suggests a more parsimonious model, in which the yeast and mammalian systems are even more similar than previously thought (FIG. 4). Get4–Get5 (in yeast) or TRC35–UBL4A–BAG6 (in mammals) is the scaffold that dynamically brings Sgt2 or SGTA into close proximity with Get3 or TRC40. This large and transient assembly is loosely defined as the TRC, within which a substrate would be sorted among these factors.

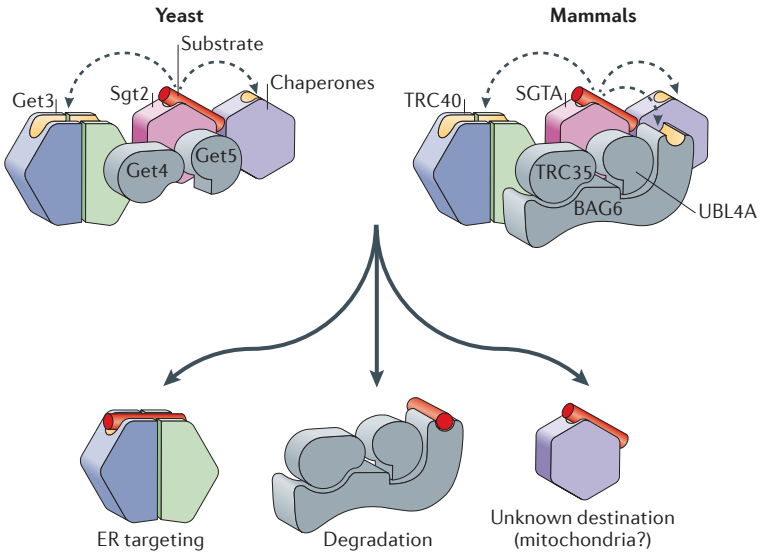

Figure 4 | **TA protein sorting by the TRC.** The transmembrane domain (TMD)-recognition complex (TRC) consists of a stable core complex (shown in grey) and several dynamically associated components. In budding yeast, the TRC core consists of Get4 and Get5, with Get4 recruiting Get3 and Get5 recruiting small Glu-rich tetratricopeptide repeat-containing 2 (Sgt2). Sgt2 can additionally recruit other chaperones. In mammals, TRC35 (which is homologous to Get4) and UBL4A (which is homologous to Get5) are in a complex with BAG6, which probably recruits SGTA (which is homologous to Sgt2). Engagement of the TRC by a substrate (probably bound to Sgt2 or SGTA) results in its sorting among any of several potential TMD-binding proteins (dashed arrows). This sorting is presumably dictated by a combination of substrate features and availability of the binding partners. The substrate can therefore emerge from the TRC bound to any of multiple binding partners, each of which imparts a specific downstream fate. Get3 or TRC40 association mediates endoplasmic reticulum (ER) targeting, whereas BAG6 binding can recruit an E3 ubiquitin ligase that mediates substrate degradation. The fates of other complexes are not understood but could include targeting to other destinations, including the mitochondria.

A committed targeting complex would be generated only if the substrate binds productively with Get3 or TRC40, a fate favoured by ER-destined TA proteins. If the substrate is unsuitable for ER targeting, it would be transferred to other factors that impart alternative fates. These might include chaperones that are specific for other destinations, such as the mitochondria, or quality control factors that mediate degradation. Indeed, recent work suggests that BAG6 is precisely such a quality control factor that can bind a range of hydrophobic substrates, recruit an E3 ubiquitin ligase and route them for proteasomal degradation[83–85].

Thus, the picture that is emerging is one of initial hydrophobic protein capture at the ribosome, followed by assembly into a highly dynamic sorting complex (the TRC) containing many potential binding partners. The substrate would then partition among the binding partners, with the final outcome depending on the specific features of the substrate. TA proteins destined for the ER would be transferred to Get3 or TRC40, whereas other hydrophobic proteins would have alternative fates. In this view, the evolution of BAG6 (which does not have an obvious yeast homologue) could have occurred to allow enhanced quality control, whereas yeast may use alternative mechanisms, including the recruitment of other chaperones, such as Hsp104 and Hsp70, both of which bind to Sgt2 (REF. 79). The precise nature of this key sorting step remains an important area of study, which will be greatly aided by the determination of the structures of complexes combined with structure-based mutagenesis studies. The hydrophobic transfer process is likely to be highly coordinated so that exposure of the TMD to the cytosol is minimized.

The substrate recognition problem is, in many ways, qualitatively different and considerably more complex for post-translational substrates than for co-translational ones. In the co-translational case, precise positioning of the SRP54 M-domain at the ribosome exit tunnel greatly simplifies the recognition problem to one of linear scanning. This not only reduces competition with other factors but also limits the degrees of freedom for both the substrate and the targeting factor. By contrast, post-translational substrates are accessible to a large number of highly abundant chaperones and co-chaperones, all of which primarily interact with proteins on the basis of their hydrophobicity. Nonetheless, effective sorting is achieved among these different factors. TA protein sorting by the TRC may therefore provide a useful paradigm for understanding protein triage among chaperone systems in general.

Protein capture at the ribosome

Although the existence of a pre-targeting factor helps to explain how substrates might be sorted and loaded onto Get3, the issue of how TA proteins are first captured on release from the ribosome remains unclear. Some insight into this initial step comes from biochemical analysis in the mammalian system, in which the BAG6 complex was observed to interact with ribosomes[78]. Its recruitment there might be mediated by TRC35 or UBL4A, the yeast homologues of which (Get4 and Get5) were found to be weakly associated with ribosomes in a proteomic analysis[86].

Remarkably, recruitment of the BAG6 complex to ribosomes was strongly favoured by the presence of a TMD inside the ribosomal tunnel[78]. The implication of this observation is that the BAG6 complex may be located favourably for initial substrate capture when the substrate is released from the ribosome. But, because TA protein release would occur very soon after the TMD is synthesized, it was unclear how the BAG6 complex could be recruited to such ribosomes in time. This seems to be aided by a TMD-dependent delay in translation termination[78].

How the termination of translation might be controlled in a substrate-specific manner remains completely obscure, although it has been observed in other contexts[87]. Similarly, precisely how sequences inside the ribosome could influence events at the surface to promote recruitment of the BAG6 complex remains a mystery. It is possible that the presence of hydrophobic sequences inside the ribosomal tunnel subtly alters ribosome conformation in a manner that is exploited by the BAG6 complex. A similar explanation has been put forward for how SRP might be recruited[88,89] and how

sequences inside the ribosome can influence its interaction with the SEC61 translocon[90–92]. Alternatively, the BAG6 complex might be recruited to ribosomes by signals that are present in the mRNA that encodes the TA protein. Indeed, studies indicate that *cis*-acting sequences in the TMD-coding region of bacterial membrane protein mRNAs can direct these mRNAs to the plasma membrane[93,94]. The initial capture step by the BAG6 complex remains to be studied in mechanistic detail, as does the apparent regulation of translational termination and the relationship between these two processes.

Targeting and release at the ER

After a TA protein is successfully loaded onto Get3 by the action of the TRC, the resulting Get3–TA protein complex must next be targeted to the ER. In budding yeast, genetic studies have indicated that Get1 and Get2, both of which are multi-spanning ER membrane proteins, are needed for targeting[65]. Furthermore, their ability to form a complex with Get3 in the absence of other factors[61,66] suggested that these three proteins could be the minimal factors required for targeting, and possibly insertion into, the ER membrane.

Insight into the role of Get1 and Get2 came from recent reconstitution studies[95,96]. Genetic and biochemical depletion and add-back experiments established that Get1 and Get2 are each indispensable for Get3-dependent insertion of the TA substrate into the membrane[95,96]. Remarkably, efficient targeting and insertion could be achieved in proteoliposomes containing only recombinant Get1 and Get2 at physiological concentrations[95]. Thus, Get1 and Get2 are both necessary and sufficient for the membrane-associated events of TA protein targeting and insertion.

The availability of a simple reconstituted system using completely purified, recombinant components permitted a detailed analysis of how these two membrane proteins interact with, and regulate the function of, Get3 (REF. 95). Interaction analysis illustrated that Get1 and Get2 associate through their membrane domains and that they each interact with Get3 via their prominent (non-homologous) cytosolic domains[95,96]. The unusual feature of a receptor that interacts with Get3 in two different ways suggested that these two interactions serve distinct functions: targeting of the Get3–TA protein complex to the ER, followed by substrate release at the ER membrane. This indeed proved to be the case, as revealed by a combination of structural and functional studies.

Insight into both steps was provided by structures of the complexes that form between Get3 and the cytosolic Get1 and Get2 receptor fragments[95,97] (FIG. 5). The structure of the amino-terminal end of the Get2 cytosolic fragment in complex with Mg^{2+}–ADP–AlF_4^--bound Get3 showed two Get2 fragments bound to equivalent sites on opposite faces of the closed Get3 dimer. Importantly, the Get3 hydrophobic groove was intact and accessible, with the N-terminal ends of Get2 tethered to the membrane by a long, flexible linker. A Get1–Get2 complex containing a structure-based mutation in Get2 that disrupts binding to Get3, was defective for TA protein insertion into the membrane[95]. These observations suggested that Get2 functions to recruit the Get3–TA protein targeting complex to the membrane.

The structure of nucleotide-free Get3 bound to the Get1 cytosolic fragment revealed two Get1 fragments bound to equivalent sites on opposite faces of the open Get3 dimer[95,97] (FIG. 5). Strikingly, each Get1 coiled-coil inserts itself between the two Get3 subunits to completely disrupt the closed dimer interface. This observation immediately suggested that Get1 functions to release substrate from Get3. Consistent with this, functional analysis showed that Get1, but not Get2, promotes substrate release[95,96]. Moreover, Get1 was unable to promote substrate release from an ATPase-deficient Get3 variant (in which Asp57 was replaced with Asn), suggesting that it functions on a Get3–TA substrate complex in which the ATP has already been hydrolysed.

The crystal structures also provided key insights into how targeting and substrate release are coordinated by the two receptor subunits. The Get1- and Get2-binding sites on Get3 are partially overlapping, and interaction analyses showed that the receptor subunits compete for binding to Get3 (REF. 95), which is consistent with a sequential handover mechanism (FIG. 5). A complex of Get3 bound simultaneously to portions of Get2 and Get1 can be detected at high concentrations by NMR, and this may represent the transient intermediate during handover[97]. Taken together, these studies suggest a model in which Get2 recruits the Get3–TA substrate targeting complex, with Get3 in a closed dimer conformation, and subsequently transfers it to Get1, which drives substrate release by disrupting the composite hydrophobic groove and stabilizing the open state of Get3.

The stoichiometry of the Get1–Get2 receptor complex remains to be established. The simplest possibility in view of the crystal structures and the symmetric Get3 dimer is that two Get1 and two Get2 subunits form a heterotetrameric assembly. The resulting high-avidity interaction of two Get1 subunits with the Get3–TA substrate complex would facilitate substrate release at physiological concentrations. Nevertheless, a heterodimeric receptor is also plausible, with Get2 binding to one side of the Get3 heterodimer and Get1 binding to the other. Evidence that this mechanism could work is provided by the finding that artificially heterodimerized Get1–Get2 cytosolic domains can mediate substrate release *in vitro*[96].

This critical step of releasing a substrate from its tightly bound targeting factor at the correct place and time is a problem faced by all targeting pathways. In the TA protein pathway, substrate release is first obligatorily 'primed' by nucleotide hydrolysis, whereas its actual release is promoted by the Get3–Get1 interaction[95,96]. This two-step mechanism is similar in concept, albeit different in details, to the SRP-mediated co-translational insertion pathway. Here, a substrate bound to SRP54 is released in two successive steps, one involving nucleotide binding and the other involving receptor interaction. The nucleotide-dependent step involves GTP binding to both SRP and its receptor to allow targeting[98,99]. The second

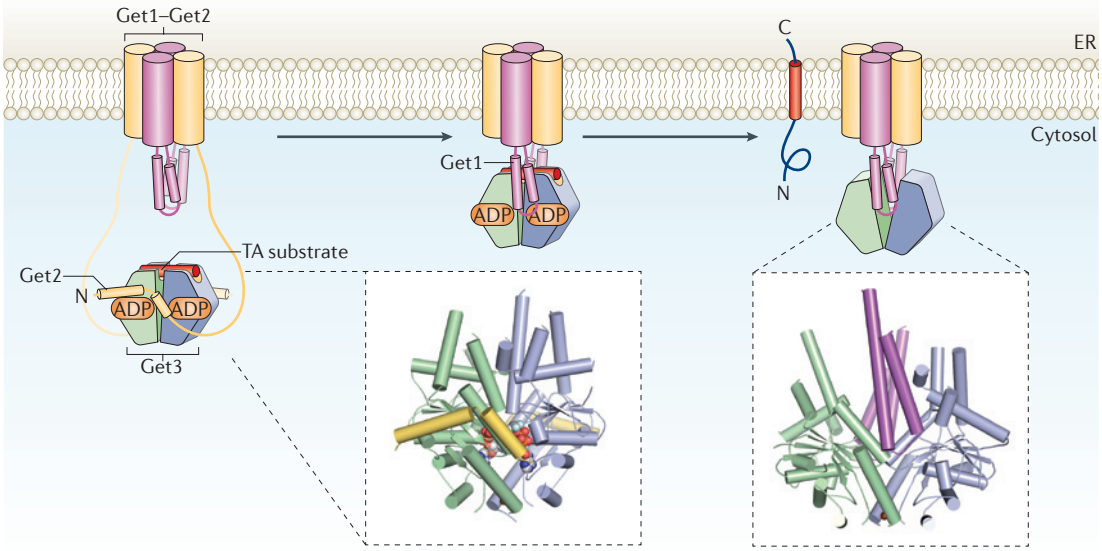

Figure 5 | **Targeting and substrate release at the ER membrane.** Nucleotide- (either ADP or ATP) and tail-anchored (TA) substrate-bound Get3 is captured at the endoplasmic reticulum (ER) membrane by the long, flexible amino termini of Get2. The Get3–TA substrate complex, now in an ADP-bound, partially closed state, is transferred to Get1, which wedges open the composite hydrophobic groove to promote TA substrate (and ADP) release. Finally, ATP re-binding dissociates the stable Get1–Get3 'post-insertion' complex to recycle Get3 back to the cytosol. Although depicted here as a stable heterotetramer, the stoichiometry and subunit composition of the Get1–Get2 receptor complex is not known. The insets show crystal structures of Mg^{2+}–ADP–AlF_4^--bound Get3 in complex with the cytosolic fragment of Get2 (left, Protein Databank (PDB) ID 3ZS9), and of nucleotide-free Get3 in complex with the cytosolic fragment of Get1 (right, PDB ID 3ZS8). C, carboxyl terminus.

step is a GTP-dependent interaction between SRP and the SRP receptor that results in structural rearrangements that expose the M-domain–signal sequence module to facilitate release to the translocon[100].

The elusive insertion step

Virtually nothing is known about how TA substrates are inserted into the ER membrane. Following its release from Get3, the substrate must avoid improper interactions (in particular, aggregation) and insert into the membrane bilayer in the correct orientation. On the basis of the rigid interaction between Get1 and Get3, the TMD is presumably released parallel to and abutting the bilayer surface. From this position, insertion requires the hydrophobic TMD to cross the polar head groups of the phospholipids and reach the hydrophobic membrane core. This could either occur 'spontaneously' (that is, without direct assistance from any factors) or the Get1–Get2 complex could chaperone the TA protein into the membrane (FIG. 6). Although *in vitro* studies show that less-hydrophobic TMDs can insert spontaneously into liposomes[44,51], most TA proteins cannot. The mechanistic basis of this final step awaits additional studies, but some insight can be gleaned from experiments done in the co-translational system.

A type I membrane protein containing a single N-terminal TMD, a short luminal domain and an extensive cytosolic C-terminal domain can be efficiently targeted to the membrane surface via the SRP pathway, but subsequent insertion fails in the absence of the SEC61 complex[101]. This indicates that simply targeting a hydrophobic TMD to the membrane surface is insufficient for insertion, and that the SEC61 complex has a crucial role. On the basis of structural and functional analysis, this crucial function is twofold[102–104]. First, SEC61 seems to directly recognize substrate TMDs via a specific binding site within its membrane domain. Second, this binding site also serves as a 'lateral gate', which provides TMDs direct access to the lipid bilayer. Thus, the TMD takes a route through the centre of SEC61 to bypass the phospholipid headgroups that otherwise preclude facile access to the hydrophobic core of the membrane.

Applying these principles to the TA protein pathway, it is attractive to speculate that Get1 and Get2, both of which are multi-spanning membrane proteins, interact directly with the TA protein's TMD to chaperone it into the bilayer. Such a recognition event could provide an additional layer of proofreading, as has been ascribed to the SEC61 complex[105–107]. More importantly, it would explain how the TMD efficiently accesses the hydrophobic core of the bilayer with minimal possibility of 'off-pathway' events such as aggregation or inappropriate interactions. One speculative model for how chaperoning could be achieved via coupled conformational changes in Get1–Get2–Get3 is depicted in FIG. 6. Alternatively, Get1–Get2 could distort the lipid bilayer in its vicinity to facilitate TMD insertion. The biochemical strategies used to study SEC61-mediated insertion in the co-translational pathway[101,104,108–110] will clearly be useful in determining the insertion mechanism of the TA protein pathway. However, distinguishing between what can happen in a simplified system and what does

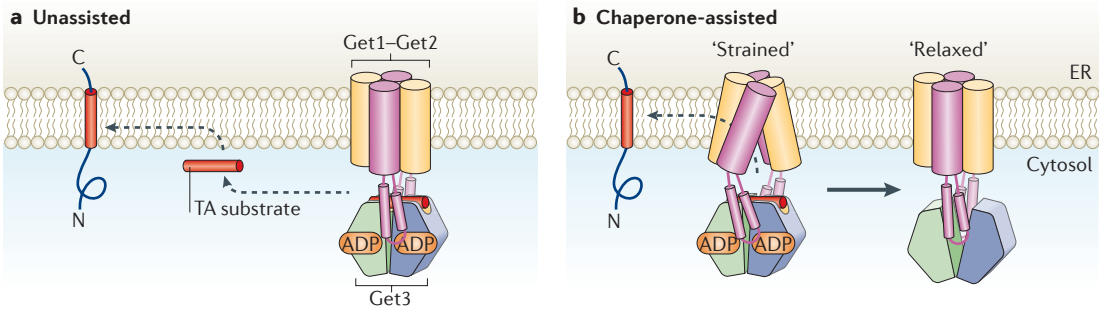

Figure 6 | **Alternative models for the insertion of TA proteins into the ER membrane. a** | After release from Get3, the tail-anchored (TA) substrate might transiently associate with the membrane surface before inserting 'spontaneously' into the lipid bilayer. **b** | Alternatively, the transmembrane domains (TMDs) of Get1 and Get2 may interact directly with the TA substrate to chaperone it into the bilayer. In the model shown here, binding of two Get1 subunits to the partially closed targeting complex results in a 'strained' configuration of the Get1–Get2 receptor complex. This arrangement might provide a hydrophobic gate through which the TMD could diffuse into the bilayer. 'Wedging open' the Get3 dimer for substrate release would then allow the Get1–Get2 receptor complex to 'relax' back into a low-energy configuration that is sealed-off from the cytosol. Thus, conformational changes in Get3 might be coupled to those of the receptor TMDs to promote TA substrate integration. C, carboxyl terminus; ER, endoplasmic reticulum; N, amino terminus.

happen in a physiological context may be challenging, as the role of Get1–Get2 in insertion might simply be to accelerate an already favourable reaction, and any consequences may only be apparent in a highly crowded context that reflects the *in vivo* situation.

Factor recycling

After the TA substrate has been released, Get3–Get1 must dissociate so that Get3 can be recycled back to the cytosol and vacate Get1 for the next substrate. The first clue for how this might be accomplished came from early studies that showed ATP-dependent release of TRC40 from the ER[55]. Structural studies provided the second clue. The crystal structure of nucleotide-free Get3 bound to the cytosolic fragment of Get1 revealed the stable, high-affinity 'post-insertion' complex[95,97]. In this open dimer conformation, the conserved hairpin loop of Get1 inserts into the Get3 active site. This interaction is both sterically and electrostatically incompatible with ATP binding, suggesting that the high intracellular concentration of free ATP could be used to displace Get3 from Get1 after substrate release. Consistent with this, interaction studies showed that ATP binding could quantitatively disrupt the Get3–Get1 interaction[95–97]. This recycling mechanism contrasts with the co-translational pathway, in which, after substrate release, the SRP–SRP receptor complex is dissociated by GTP hydrolysis[111].

After release from Get1, what prevents Get3–ATP from re-binding to Get2 in a 'dead-end' complex that cannot recruit a new TA substrate? Previous studies identified Get3 mutations that disrupt ATP-dependent binding to Get4 (REF. 80). Remarkably, these mutations map to the overlapping Get1- and Get2-binding sites on Get3. Thus, by competing for the same site as the Get1 and Get2 receptor complex, Get4 binding could sequester the recycled Get3–ATP complex in the cytosol and promote another round of substrate loading by the TRC. This model is consistent with the observation that distinct Get1–Get2–Get3 and Get3–Get4–Get5 complexes can be isolated from fractionated yeast[66], and provides an elegant mechanism for spatially regulating Get3 activity in the cytosol and at the ER membrane.

The ATPase cycle

Biochemical, genetic and structural studies of the various steps in the TA protein pathway have begun to clarify the role of ATP binding and hydrolysis by Get3 in the insertion cycle. As is the case for SRP in the co-translational pathway, the overarching theme is that the nucleotide state directly influences the conformation of Get3, which in turn regulates its interactions with the TA substrate, targeting cofactors and receptors at the ER membrane. Some of these interactions subsequently change the nucleotide state. This allows Get3 to proceed undirectionally through its cycle of conformational changes and thereby selectively bind substrate in the cytosol and release it at the membrane.

Under physiological conditions, Get3 in the cytosol is probably in an ATP-bound closed state[95]. The closed conformation preferentially incorporates into the TRC owing to the nucleotide-dependence of the Get3–Get4 interaction[80,96]. Thus, substrate loading onto Get3 via the TRC requires ATP binding. Once loaded, the TA substrate bridges the helical domains across the hydrophobic groove of Get3. This locks Get3 into a closed conformation, and traps the nucleotide in the dimer interface. It is possible that substrate binding stimulates ATP hydrolysis by Get3, which would weaken its interaction with Get4 and promote disengagement from TRC. This would generate a committed ADP-bound Get3–TA substrate complex that is stabilized in a closed conformation by the tightly bound substrate.

This targeting complex would be captured at the membrane by Get2 and then transferred to Get1. Although targeting seems 'agnostic' to the nucleotide state of Get3, the subsequent transfer reaction absolutely requires ATP

to have been hydrolysed[95,96]. This is probably because the binding site for Get1 is partially buried in the fully closed ATP-bound state of Get3 (REF. 95). Get1 binding causes Get3 to fully open, thereby favouring substrate release. Once Get3 opens, the ADP is no longer trapped and probably dissociates rapidly. The re-binding of ATP to Get3 dissociates it from Get1. This effectively means that Get1 acts as a nucleotide exchange factor that displaces ADP (via opening of the Get3 dimer) and allows its replacement with ATP.

Whereas most parts of the ATPase cycle have strong experimental support, the timing of ATP hydrolysis remains unclear. It must occur at a point after the substrate binds to Get3 and before the interaction of this protein with Get1. Although it is attractive to posit that hydrolysis stimulated by either substrate or Get2 interaction provides an additional checkpoint, this may not be necessary. In fact, a slow rate of intrinsic hydrolysis might provide a mechanism for kinetic proofreading. In this model, loosely bound substrates would dissociate before hydrolysis, thereby preventing their membrane-localized release by Get1. This might reduce inappropriate targeting of mitochondrial TA proteins (which generally have TMDs of lower hydrophobicity than ER-directed TA proteins)[47–49,77] to improve the overall fidelity of sorting. Similar kinetic proofreading mechanisms have been uncovered in the SRP pathway and are thought to maximize the sorting efficiency of an otherwise promiscuous interaction between SRP and various proteins[112].

Future challenges and perspectives

Since the discovery of the TA protein insertion pathway in 2007, rapid advances have been made through the application of genetic, biochemical and structural approaches. Over the past 5 years, all of the core components have been identified, and a general mechanistic understanding of the pathway from ribosome to the ER is now in hand. But the details of many key steps that take place in the cytosol and at the membrane remain a mystery. For example, how does the TRC selectively interact with cytosolic ribosomes that contain a TMD in the exit channel? Structural studies are needed to identify the ribosomal binding site of the TRC, and to determine how the TRC senses the presence of a TMD in the exit channel. After this initial capture step, what is the mechanism for TA substrate transfer from the TRC to Get3? Although crystal structures exist for Get4 and portions of Get5 and Sgt2 (REFS 80,113–115), mechanistic insight awaits structural analysis of the intact TRC. More broadly, how does the TRC sort different hydrophobic substrates between its multiple hydrophobic region-binding factors (including the chaperones BAG6, Sgt2, Get3, HSP70 and possibly others)? This critical checkpoint determines the fate of the hydrophobic substrate: insertion into the ER membrane (via Get3), insertion into the mitochondrial outer membrane (by an unknown mechanism), or ubiquitylation (in a process involving BAG6) and proteasomal degradation for misfolded membrane proteins. Defining the mechanism of this process is therefore an important future goal.

The biochemical and structural framework established over the past few years also sets the stage for detailed mechanistic studies of events at the ER membrane. Determining the structure and organization of the Get1–Get2 receptor complex will be critical for understanding insertion. Is the receptor a stable heterodimeric, heterotetrameric or higher order assembly? Or are there temporal variations in its stoichiometry and subunit composition? Similarly, the order and timing of Get3–substrate interactions with the receptor have not been directly established. Most significantly, almost nothing is known about how the TA substrate inserts into the bilayer after its release from Get3. Does it insert spontaneously? Or is it actively chaperoned by the conserved TMDs of Get1 and Get2? Answers to these questions will require high-resolution structural analysis of Get1–Get2–Get3 complexes trapped at different stages of this cycle. This promises to be technically challenging, but the goal is in reach now that robust expression systems for functional Get1 and Get2 proteins are in hand.

Finally, there may be much to learn from studying TA protein biogenesis in other organisms. The recent discovery of a conserved Get3 orthologue in archaea indicates that the post-translational TA pathway is more broadly conserved than previously appreciated[116,117]. No obvious sequence homologues have been identified for other cytosolic components (for example, BAG6, Sgt2, Get4 or Get5), but functional data are consistent with the presence of an orthologous integral membrane receptor in archaea[117]. If such a receptor exists in archaea, it shares only limited sequence homology with the yeast receptor subunits, Get1 and Get2. There are also intriguing evolutionary differences between the yeast and mammalian pathways. The absence of a BAG6 homologue in yeast suggests increasing complexity in the pre-targeting machinery of higher eukaryotes. And, whereas the human Trp-rich basic (WRB) protein shows sequence and functional homology to yeast Get1 (REF. 118), no obvious sequence homologue to Get2 has been found in higher eukaryotes. Thus, the identification and characterization of components in the archaeal and higher eukaryotic pathways promises new insight into the mechanism of TA protein targeting and insertion.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
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