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Review

Targeting pathways of C-tail-anchored proteins ☆

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ABSTRACT

A large group of diverse, functionally important, and differently localized transmembrane proteins, share a particular membrane topology, consisting of a cytosolic N-terminal region, followed by a transmembrane domain close to the C-terminus. The C-terminal membrane anchor of these tail-anchored (TA) proteins generally represents the sole targeting determinant, and becomes available to targeting factors only after release of the finished polypeptide from the ribosome. Hence, TA proteins do not have a chance to interact co-translationally with Signal Recognition Particle and are delivered post-translationally to all target membranes, including the ER. Recent work has demonstrated the existence of different biogenetic pathways for TA proteins. Notably, some are able to efficiently translocate their C-terminus across protein-free bilayers without the participation of any membrane or cytosolic protein, while others require assistance from cytosolic chaperones and membrane receptors. In this review, we summarize current knowledge on the different insertion pathways, with emphasis on a recently discovered chaperone system that operates in fungi as well as in higher eukaryotes to deliver TA proteins to the ER (called Guided Entry of Tail-anchored Proteins (Get) system and Transmembrane Recognition Complex (TRC), in yeast and mammals, respectively). We suggest that the final insertion step of TA proteins does not require membrane proteins, but that different competing chaperone systems ensure precise delivery to defined targets while preventing inappropriate insertion into otherwise permissive bilayers. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

Abbreviations: b5, cytochrome b5; COE, chloroplast outer envelope; ER, Endoplasmic Reticulum; Get, Guided Entry of Tail-anchored Proteins; Hsc, Heat Shock Cognate Protein; Hsp, Heat Shock Protein; OMM, outer mitochondrial membrane; RRL, rabbit reticulocyte lysate; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SRP, Signal Recognition Particle; Syb, synaptobrevin; TMD, transmembrane domain; TOM, translocase of the outer membrane; TRC40, Transmembrane Recognition Complex subunit of 40 kDa; Ubl, Ubiquitin like domain

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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1. Introduction

Tail-anchored (TA) proteins are transmembrane polypeptides characterized by an N-terminal functional cytosolic region anchored to the lipid bilayer by a single transmembrane domain (TMD), followed by a lumenal polar sequence no longer than 30 residues [1]. Bioinformatic analyses indicate that TA proteins are well represented

in all three domains of life, where they carry out a variety of essential functions that benefit from, or require, membrane anchorage, such as membrane fusion in vesicular transport, protein translocation, regulation of apoptosis, storage of transcription factors, and enzyme catalysis [2–5].

Because the C-terminal TMD constitutes the only membrane-targeting sequence and because it emerges from the ribosome tunnel only after termination of translation, TA proteins must insert into all their target membranes—endoplasmic reticulum (ER), outer mitochondrial, outer chloroplast, peroxisomal, and prokaryotic cytoplasmic membranes—by post-translational pathways. Other, non-TA, membrane proteins of mitochondria, plastids and peroxisomes also insert by post-translational mechanisms, however, proteins targeted to the ER- and the bacterial cytoplasmic membrane generally use the co-translational pathway mediated by Signal Recognition Particle (SRP) [6]. TA proteins constitute a clear-cut exception to this rule. The unique mechanisms by which the members of this group of functionally important proteins achieve their final subcellular localization have recently attracted a great deal of interest, and constitute the subject of this review.

A number of comprehensive review articles on TA proteins have been published in the recent and less recent past [1,7–12], and the reader is referred to these papers, as well as to the publications reporting bioinformatic analyses [2–5,12], for detailed information on the localization and functions of TA proteins in different organisms. In this review, we will focus on recent progress on TA protein biogenesis, discussing unanswered questions and attempting to give a unified picture of the general mechanisms underlying their targeting to—, and integration into membranes.

As mentioned above, TA proteins can be inserted from the eukaryotic cytosol into a number of target membranes, i.e., the ER, the outer mitochondrial membrane (OMM), the Chloroplast Outer Envelope (COE), and the peroxisomal membrane. Examples of TA proteins specifically targeted to these different membranes are given in Table 1. The ER represents the major destination of TA proteins [3], and from this initial insertion site many are then exported to different compartments of the endo-membrane pathway (see Table 1). We will first discuss recent advances in our understanding of insertion mechanisms of ER-targeted TA proteins, then summarize what is known on their targeting to other organelles, and finally attempt to give a conceptual framework in which to fit our present knowledge on TA protein targeting.

2. Insertion of ER-targeted TA proteins

Although it was realized early on that TA proteins are directed to the ER only after their release from the ribosome, it was considered possible that they might translocate their C-terminus by post-translational engagement of the Sec61 translocon. However, work with a number of substrates in different systems (mammalian and yeast, the latter both *in vivo* and *in vitro*) has excluded a role for Sec61 in TA protein transmembrane integration [13–16]. It has instead become apparent that more than one Sec61-independent pathway operates in ER targeting of TA substrates. More specifically, *in vitro* studies have revealed the existence of an *unassisted* pathway by which substrates with weakly hydrophobic TMDs spontaneously integrate into the lipid bilayer, and *chaperone-mediated*, energy-requiring pathways, by which most ER-targeted TA substrates reach their destination.

2.1. Unassisted insertion of ER-targeted TA proteins

Mammalian cytochrome b5 (b5) is the most thoroughly investigated ER TA protein capable of unassisted insertion. Early studies indicated that purified b5 could associate with pure phospholipid liposomes with a hairpin topology (N and C-terminus on the outside of the vesicle; [17]). Much later, our laboratory, using stringent proteolysis protection assays for *bona fide* transmembrane integration of TA proteins, demonstrated that b5, translated *in vitro* in rabbit reticulocyte lysate (RRL) translocates its C-terminus across pure lipid bilayers as rapidly and as efficiently as across microsomal membranes [16]. This work left open the possibility that chaperones of the RRL might be required to keep b5 in an insertion competent form. To investigate this possibility, we expressed b5 as fusion protein and purified it free of chaperones. A complete lack of influence of RRL on the extent and kinetics of transmembrane integration of this protein into pure lipid vesicles demonstrated unequivocally that, at least *in vitro*, b5 can translocate its C-terminus across the lipid bilayer in the absence of any membrane or cytosolic protein, and that these do not even facilitate the insertion process [18]. Interestingly, however, the presence of even low concentrations of cholesterol in the vesicles sharply inhibits the unassisted integration process [16].

Since it was known that other TA proteins do require ER membrane protein(s) and energy for their insertion [13,19], we investigated what feature of b5 is responsible for its capacity to insert without assistance. Production of chimaeric proteins and mutagenesis experiments

Table 1
Examples of TA protein targeting.

Target membrane	TA protein	Function	Notes	References
ER	Sec61β	Protein translocation		[23,25]
	Ramp4	Protein translocation		[26]
	Synaptobrevin-2	SNARE required for synaptic vesicle exocytosis	Also known as VAMP (Vesicle Associated Membrane Protein)-2. Transported down the secretory pathway from the ER to synaptic vesicles in neuronal cells	[13]
	Sed5	Golgi SNARE (Yeast orthologue of mammalian syntaxin 5)	Transported from the ER to the Golgi complex	[29]
	Cyt b5	Lipid metabolism in the ER		[62,74]
OMM	Small TOM proteins	Protein translocation		[73]
	OMb5	Enzymatic	Mitochondrial isoform of cyt b5	[62,74]
	VAMP-1B	SNARE of unknown function	Splicing variant of ER-targeted isoform (VAMP, or synaptobrevin-1A)	[61]
	Bcl-XL, Bak	Regulation of apoptosis		[68]
COE	*A. thaliana* cyt b5 isoform At1g26340	Enzymatic		[75]
	Outer Envelope Membrane Protein 9	Unknown		[77]
	Toc 33, 34	Protein translocation		[76,77]
Peroxisomes	Pex26	Peroxisome biogenesis		[80]
Dual targeting: ER and OMM	Bcl-2	Regulation of apoptosis		[81]
Dual targeting: OMM and peroxisomes	Fis1	Mitochondrial and peroxisomal fission		[59]

demonstrated that the ability for unassisted insertion is conferred by moderate hydrophobicity of the TMD [20]. Synaptobrevin 2 (Syb2) is a well-known TA substrate incapable of unassisted insertion. Substitution of Syb2's TMD with the one of b5 converted Syb2 into an unassisted substrate, and conversely, b5 was converted into an assisted substrate by substituting its TMD with the one of Syb2. Furthermore, point mutations decreasing or increasing TMD hydrophobicity of Syb2 and b5 respectively favoured or inhibited their unassisted insertion, in agreement with the conclusion that TMD hydrophobicity is the main determinant for access to, or exclusion from, the unassisted pathway [20]. Finally, the hydrophobicity hypothesis also proved to have a predictive value: two ER-targeted TA proteins, one with TMD hydrophobicity close to the one of b5 (Protein Tyrosine Phosphatase 1B—PTB-1B) and one with a more hydrophobic TMD (Vesicle Associated Membrane Protein Associated Protein-B—VAP-B) were analyzed and found to insert by the unassisted and by an assisted pathway respectively, as expected [20,21].

Regardless of how unassisted proteins are targeted *in vivo* (discussed in Section 4), the demonstration of efficient spontaneous transmembrane integration of TA proteins with moderately hydrophobic TMDs has implications for the understanding of the more common assisted pathways. The requirement for chaperones for most ER-targeted TA proteins is most likely related to the propensity of hydrophobic sequences to aggregate in water, and also to the difficulty for a hydrophobic sequence to cross the barrier of phospholipid polar headgroups at the interface between the bilayer and the surrounding aqueous phase. However, it is conceivable that all TA proteins, once delivered to the appropriate membrane, can translocate their C-terminus directly across the bilayer without the need for any insertion machinery. In this view, the problem of TA protein biogenesis is reduced to how the cell manages to deliver different substrates to the correct target membrane in an insertion competent form.

2.2. Chaperone-mediated pathways for ER-targeted TA proteins

Three chaperone-mediated pathways have been described so far (reviewed in [11]): one mediated by SRP, functioning in an unusual post-translational mode, one by the Hsc70/Hsp40 couple, and one by a novel ATPase previously known as Asna1 (arsenical pump-driving ATPase protein) in mammals and Arr4p in yeast. Following the demonstration of its role in TA protein delivery to the ER, it was renamed Transmembrane Recognition Complex subunit of 40 kDa (TRC40) or Guided Entry of Tail-anchored proteins 3 (Get3) in mammals and yeast, respectively.

The involvement of SRP and Hsc70/Hsp40 was suggested by probing cross-linked adducts of TA substrates to RRL proteins with antibodies against these chaperones. A facilitatory role of the identified chaperones on insertion was then demonstrated using truncated TA substrates released by puromycin from purified ribosome-nascent chain complexes [22,23]. In addition, Hsc70 was shown to interact with the TMD of a TA protein in plants, and to facilitate its *in vivo* targeting to the nuclear envelope [24].

TRC40 was identified by unbiased examination of the interacting partners of naturally terminated TA substrates [25,26]. Several considerations indicate that the pathway mediated by this ATPase is the most important one for targeting TA proteins to the ER. First, cross-linking studied under a variety of conditions demonstrate that, *in vitro*, TRC40 is the major cross-linking partner of a variety of TA substrates (see [25], supplemental note for a discussion); second, depletion of the SRP receptor from microsomal membranes is generally without effect on TA protein insertion; furthermore insertion is generally ATP- and not GTP-dependent [13,14,25], again suggesting that SRP does not play an important role; third, in the RRL, small molecule inhibition of the Hsp40/Hsc70 complex has no effect on assisted TA substrates [27], indicating that these chaperones are dispensable for insertion into the ER; in contrast, addition of an ATPase-deficient mutant of TRC40 to *in vitro* translation samples [25] or antibody-mediated depletion of the ATPase from the RRL [18,28] both strongly inhibit insertion of chaperone-requiring TA substrates; finally, deletion of the yeast homologue Get3 has demonstrated the importance of this ATPase in TA protein insertion *in vivo* [29]. We will devote the remaining part of the section on ER-targeted TA proteins to this novel chaperone system.

2.2.1. Discovery of the TRC40/Get pathway

TRC40 and Get3 are P-loop ATPases that belong to a family of GTPases (named SIMBI for signal recognition particle, MinD and BioD), some of which have acquired ATPase activity during evolution [30]. The bacterial homologue, ArsA, is involved in heavy metal resistance [31], and may, together with its membrane-embedded partner Ars B, effect arsenite extrusion across the cytoplasmic membrane [32]. The eukaryotic homologues, however, do not have an oxyanion binding site, and their exact function remained unclear until recently.

As summarized in the first paragraph of Section 2.2, biochemical experiments carried out in the RRL by Stefanovic and Hegde [25] first led to the discovery of TRC40 as a chaperone that binds the TMD of TA proteins and that plays a prominent role in TA protein biogenesis. In mammals, deletion of TRC40 is embryonic lethal [33], suggesting that, unless TRC40 has some other yet unknown function, at least one essential TA protein critically depends on TRC40 for its targeting/insertion.

The work of Stefanovic and Hegde [25] was largely confirmed by a subsequent study [26], which, in addition, reported redox sensitivity of the TRC40-dependent pathway. Both papers reported no, or very weak association of the non-assisted substrate b5 with TRC40, confirmed by its insensitivity to dominant negative TRC40 [25] and to TRC40 depletion [18].

In parallel with these studies in the mammalian system the Get3 pathway was characterized in yeast [29,34,35]. At variance with the situation in mammals, Get3 (previously known as Arr4) is not an essential gene in *Saccharomyces*, although its deletion confers increased sensitivity to stress agents, such as heat or metals [36,37] and causes defects in meiotic spore formation [38]. Based on a large-scale genetic interaction map of the secretory pathway, obtained through epistatic miniarray profiling of yeast deletion strains, Schuldiner et al. [34] uncovered a strong interaction between *get3* and two other genes, which were called *get1* and *2*, and confirmed previous work showing that the three gene products are physically associated. Initially it was thought that the complex is involved in retrograde transport from the Golgi complex to the ER (hence the name, Golgi ER trafficking 1–3). When the role of the mammalian orthologue in TA protein targeting was discovered, an analogous function of the Get 3 system was demonstrated in yeast both *in vivo* and *in vitro*, and, accordingly, the meaning of the acronym was changed to "Guided Entry of Tail-anchored proteins". More specifically, the following points were demonstrated: i) in agreement with previous work [38], the transmembrane proteins Get1 and 2 constitute the ER receptor for Get3; ii) Get3 interacts with the TMD of a number of ER-targeted TA proteins, which are in large part displaced to the cytosol in the absence of Get3; iii) in the absence of Get1/2, these TA proteins accumulate in cytosolic puncta which contain Get3, indicating that the substrates remain trapped in an unproductive complex with Get3; this phenotype correlates with the aggravated growth defect of ΔGet1/2 strains in comparison with strains in which all three of the Get proteins are absent; iv) Get deletion causes severe impairment in translocation of the C-terminus in *in vitro* translocation assays; v) in the absence of the Get complex, a subset of ER-directed TA proteins mislocalize to the mitochondria, suggesting that the Get pathway, in addition to a positive role in the delivery of TA substrates to the ER, also hinders them from inappropriate localization to alternative organelles; vi) finally, the pleiotropic, and previously poorly understood, effects of *get3* deletion can each be explained by defective insertion of specific TA proteins.

In subsequent screens aimed at characterizing genes required for protein folding in the ER [35] or for retrieval of resident ER proteins from the Golgi complex [39], two further genes—*yor164c* and *mdy2*—that interact with *get3* were identified, and accordingly renamed *get4* and *5*, respectively [35]. Get5/Mdy2 contains a ubiquitin-like-domain (Ubl) and forms a complex with Get4. Get3 can be isolated from the cytosol in a complex with Get4/5, and Δget5/Mdy2 strains have similar defects in TA protein targeting as the strains deleted in the other members of the pathway. Since Get4 and 5 have been found to weakly associate with ribosomes [40], it was suggested they could capture TA proteins upon their release from the ribosome and hand them over to Get3 [35].

Recent biochemical studies in the mammalian system also support the concept of a ribosome-associated complex acting upstream to TRC40. Two groups [28,41] discovered an unexpected role for Bat3 (HLA-B associated transcript 3, also known as Scythe or Bag6), a protein that contains an N-terminal Ubl, a nuclear localization sequence, and a C-terminal BAG domain and that has been implicated in a variety of biological processes (see Discussion in [28]). The first study [28] demonstrated that immunodepletion of Bat3 from the RRL severely inhibits insertion of assisted substrates, but Bat3 alone is not capable of supporting insertion in the absence of TRC40. Sucrose gradient fractionation showed that complexes enriched in TRC40 are more efficient than those enriched in Bat3 in delivering a TA substrate to the membrane, suggesting that Bat3 acts upstream to TRC40. The second study [41] demonstrated that Bat3 exists in a complex with the mammalian homologues of Get4/5 (TRC35 and Ubl4A respectively), and that it promotes binding of TA substrates to TRC40 after their release from the ribosome. Very interestingly, the complex appears to specifically recognize ribosomes that carry a TMD within the tunnel, so that it is positioned to capture the substrate immediately upon its release. In addition, the tail anchor in the ribosome tunnel slows the process of chain termination, thus allowing more time for recruitment of Bat3/TRC45/Ubl4A. These findings might explain how the complex can do its job, even if its intracellular concentration is much lower than that of ribosomes. Indeed, in yeast, the number of copies of Get4 and 5 per cell are estimated at 5400 and 6500, respectively (Saccharomyces Genome Database) in the face of ~300 000 ribosomes/cell. If the complex were to randomly sample all ribosomes, the chances of its being on the right ribosome at the right time would be extremely low.

A final additional component, Sgt2, has recently been assigned to the Get pathway in yeast. Sgt2 is a tetratricopeptide repeat-containing protein, which interacts genetically and physically with Get4/5 [42–45]. This interaction involves Get5/Mdy2's Ubl domain. Sgt2 has a mammalian homologue (SG2A; [46]), and also interacts with members of the Hsp70 chaperone family. Deletion of Sgt2 in yeast causes a mislocalization of the TA protein PEX15 similar to that observed when Get3 is absent [44], however, its precise role in the Get pathway remains to be elucidated [28,45].

Whereas Get3–5 are conserved between yeast and mammals, this is not the case for all components of the pathway. Notably, Bat3 does not appear to have a yeast orthologue, and, *vice versa*, no mammalian orthologue of Get2 has been identified, and the involvement of the Get1 homologue (Tryptophan Rich Basic Protein, WRB) as TRC40 receptor on the ER has not been demonstrated. Strikingly, however, a preformed complex between fungal Get3 and a mammalian TA protein (Ramp4) is capable of efficiently releasing the substrate to mammalian ER microsomes, indicating that the as yet undiscovered mammalian receptor functionally interacts with the fungal chaperone [47]. A summary of the components of the Get/TRC40 pathway so far identified is provided in Table 2.

In summary, a considerable amount of work indicates that the Get/TRC40 pathway plays a prominent role in TA protein targeting to the ER. However, the finding that *S. cerevisiae get* genes are not essential indicates that Get substrates do have salvage pathways by which they can reach the ER. These could be provided by the non-assisted (Section 2.1), and/or by the SRP and Hsp40/Hsc70 (described at the beginning of Section 2.2) pathways. The involvement of Hsp40 is suggested by the observation that in *S. cerevisiae* deletions of a component of the Get pathway and of the Hsp40 protein YDJ1 are more deleterious when combined than when present as single mutations; this is to be expected if each of the two chaperone systems can partially compensate for the loss of the other one [45].

2.2.2. Structural studies and models for the mechanism of Get3-mediated insertion of TA proteins

Following the discovery of the role of TRC40/Get3 in TA protein targeting, a lot of research has been aimed at unraveling the mechanism of action of this novel ATP-dependent chaperone, and in less than half a year, five structural studies on Get3 have been published [47–51]. Based on this structural information and on parallel biochemical experiments, a number of models for the Get3 functional cycle have been proposed, but at present it is difficult to reconcile some of the conflicting data, and exactly how Get3 couples ATP hydrolysis to TA binding and release is not yet clear.

The salient features that emerge from all the structural studies are: i) Get3 exists as a homodimer. At the interface between the two monomers, a zinc ion is coordinated by two Cys residues on each monomer, to obtain a composite zinc finger: these two Cys residues are essential for Get3 function; ii) each Get3 monomer is composed of an ATPase domain, which contains all the features generally found in G-type hydrolases, and an α-helical subdomain, which appears to be highly dynamic, and which is unusually rich in Met residues. Mutational and amide hydrogen exchange measurements indicate that this region contains the TA binding site; conformational changes in this region affect the activity of the ATPase domain, indicating that the two regions are functionally linked; iii) different conformational states have been captured in the five structural studies, categorized as completely closed,

Table 2
Components of the Get/TRC40 pathway.

Protein		Role in pathway	References
S. cerevisiae	Mammals		
Get3	TRC40	ATPase that binds the transmembrane domain and delivers TA substrates to the ER membrane.	[25,26,29]
Get1	Tryptophan Rich Basic Protein (WRB)	Transmembrane protein of the ER. In *Saccharomyces* the Get1/2 complex constitutes the Get3 Receptor; a similar role for the mammalian homologue has not been demonstrated.	[29]
Get2	–	Transmembrane protein of the ER. In *Saccharomyces* the Get1/2 complex constitutes the Get3 Receptor.	[29]
Get4	Conserved Edge expressed protein (cee)/TRC35	Get4/5 complex is thought to deliver TA proteins from the ribosome to Get3. In mammals, it is associated with a third component, Bat3.	[35,39,41]
Get5/Mdy2	Ubl4A	Ubl domain containing protein. Get4/5 complex is thought to deliver TA proteins from the ribosome to Get3. In mammals, it is associated with a third component, Bat3.	[35,39,41]
–	Bat3	Ubiquitin-like and BAG domain containing protein. Acts upstream to TRC40.	[28,41]
Sgt2	SGTA	Tetratricopeptide repeat peptide-containing protein that interacts with Get4/5. Precise role in TA protein biogenesis not understood.	[43–45]

closed, and open. The open-to-closed transition occurs about a hinge point centered on the coordinated zinc ion. The completely closed conformation is seen in the ADP-AlF_4-bound protein that represents the transition state primed for ATP hydrolysis [48]. The closed conformation is seen in both the pre-hydrolysis (AMPPNP-Mg^{2+}) and the post-hydrolysis (ADP-Mg^{2+}) states [47]. Open conformations are observed in the nucleotide-free state or in the protein with bound ADP but no Mg^{2+} [48,49,51].

In the completely closed conformation (ADP-AlF_4-bound), the two subunits present a large dimer interface surface area; the rearrangement of the α-helical subdomain results in the generation of a composite hydrophobic groove that most likely provides a binding site for the TA and which appears well suited to accommodate a hydrophobic stretch of ~20 residues, as found in many ER-targeted TA proteins (Fig. 1). The abundance of methionine residues within the groove recalls a similar feature in the M (signal peptide binding) domain of SRP54 and may contribute to the ability of Get3 to accommodate diverse tail anchors. In the open conformation, the two subunits are splayed apart so that a large cleft is interposed between the two α-helical subdomains and the composite hydrophobic groove is disrupted (Fig. 1). In the closed (AMPPNP-Mg^{2+} or ADP-Mg^{2+}-bound) state, the hydrophobic groove observed in the ADP-AlF_4-bound protein is not fully assembled.

From these studies it appears that ATP hydrolysis is possible only when the TA cargo is bound. However, how TA binding and release and ATP hydrolysis are coordinated is still unclear. Initially, it was thought that release to the membrane is triggered by ATP hydrolysis [52]. However, this model is not compatible with recent studies, in which a TA substrate was expressed in *E. coli* together with Get3 [47,51] or TRC40 [53]. A nucleotide-free complex containing the chaperone and its bound substrate could be isolated from the bacterial lysate. Subsequent release of the substrate to ER microsomes was triggered by ATP, but also, to a lower, but still considerable extent, by ADP and by a non-hydrolysable ATP analogue. In consideration of these data, and of the finding that the ADP-Mg^{2+} structure is closed, Bozkurt et al. [47] proposed that the ATP hydrolysis step is required for the release of Get3 from its membrane receptor rather than for delivery of the TA substrate to the bilayer. The situation is further complicated by the report of an open, nucleotide-free, conformation of Get3 that presents a possible TA binding site ([50,51]; Fig. 2), and by the isolation of complexes between a bacterially co-expressed TA substrate and ATPase-deficient Get3 mutants [51]. It should be kept in mind that complex formation between overexpressed proteins in the bacterial cytosol may not be reflecting events occurring under physiological conditions. This *caveat* is reasonable, also considering that, in yeast, TA delivery to Get3 depends on the presence of Get4/5 and these proteins were not co-expressed in the experiments in bacteria. Four different models that have been proposed for Get3/ATP-driven insertion of TA proteins are depicted in Fig. 2.

There is also work in progress on the Get4/5 complex. Three crystal structures of Get4 have been published so far [45,54,55]. In two of these structures [45,55], Get4 is in a complex with the N-terminal fragment of Get5/Mdy2. Get 4 is an elongated protein, composed of 14 pairwise arranged α-helices (α-solenoid fold) and an additional α-helix close to the C-terminus. A combination of structural, physicochemical, and biochemical studies indicate that the Get4/5 complex exists as a homodimer [55] and that each Get4 molecule forms a bridge between Get5/Mdy2 and Get3. These associate with Get4's C- and N-terminal regions, respectively. Get3 binding is stimulated by addition of ADP or ATP, suggesting that it binds in the closed state, and adding complexity to the role of the ATP hydrolysis cycle in TA protein targeting [55]. The Get5/Mdy2 structure, apart from the N-terminal domain in complex with Get4 has not been resolved yet. However, mutational analysis indicates that its C-terminal domain is responsible for homodimerization of the complex [55]. Intriguingly, the N-terminal domain of Get5/Mdy2 that interacts with Get4 is not present in the mammalian homologue, Ubl4A. Perhaps the presence of Bat3 in the mammalian complex

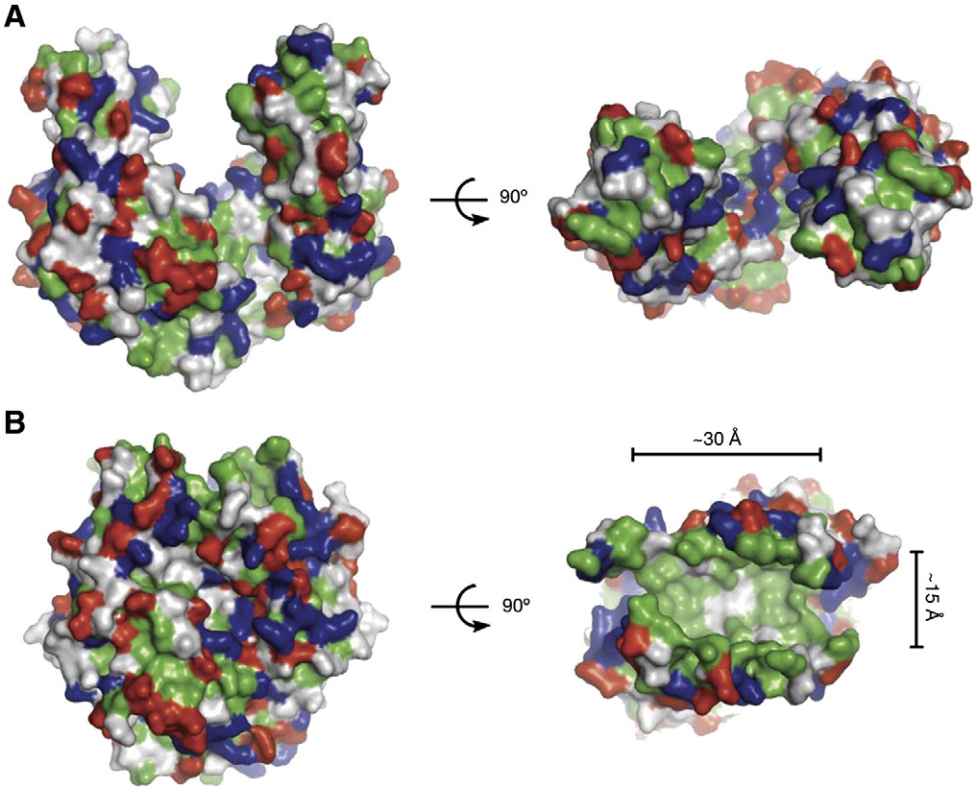

Fig. 1. Closed and open states of Get3 dimer. A and B show the open (nucleotide-free) and closed (ADP-AlF_4-bound) state, respectively. Hydrophobic residues are colored green; positively and negatively charged residues are in blue and red, respectively. The dimensions of the composite hydrophobic groove are indicated on the right side of panel B. Reproduced with permission from [48].

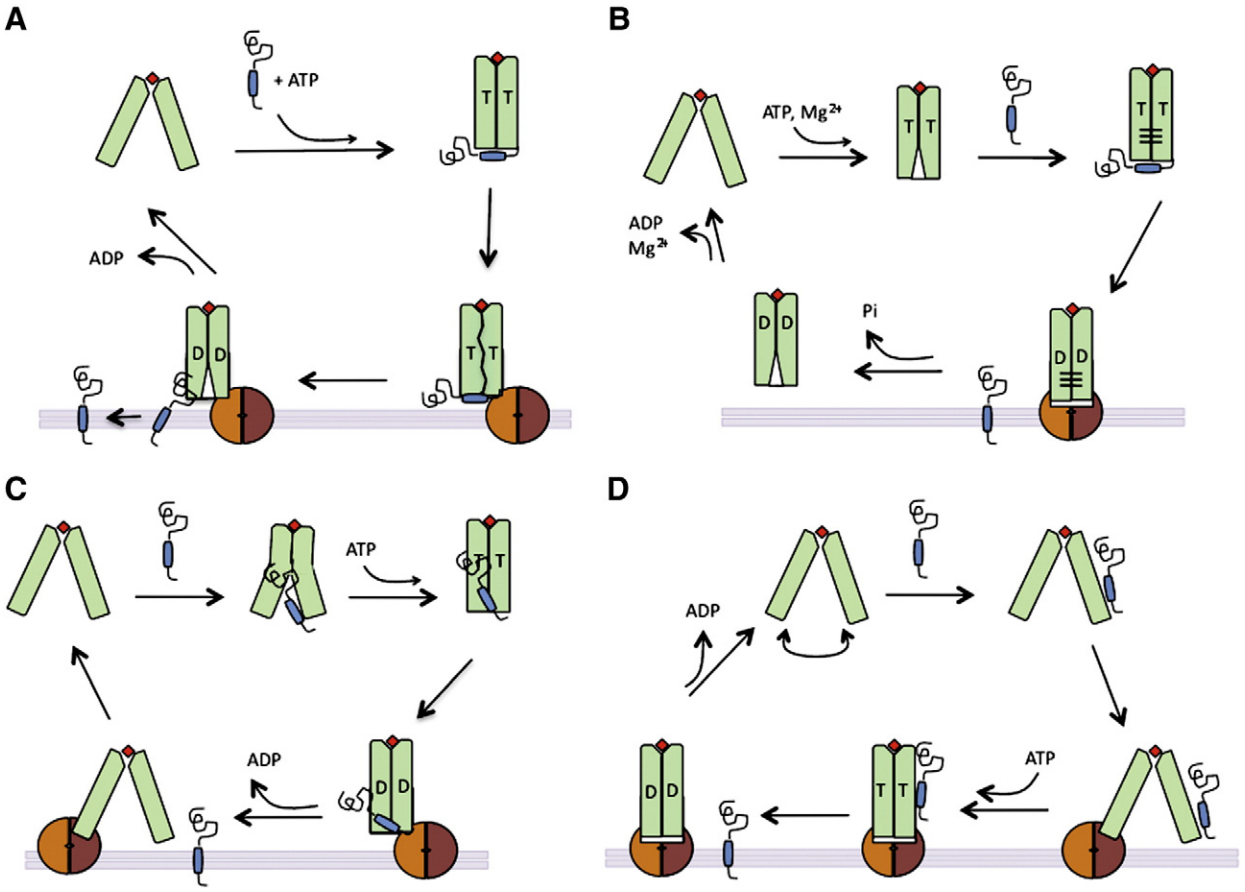

Fig. 2. Four different models for the Get3 functional cycle. Models depicted in [44,45,47,48] have been redrawn in a uniform style. The Get3 dimer is in green, and the Get3 receptor at the ER is in two shades of brown. The red diamond indicates a Zinc ion. T and D indicate the presence of bound ATP or ADP, respectively. A: ATP and TA binding are coupled, and ATP hydrolysis, triggered upon membrane binding of the complex, is required for release of the TA to the bilayer [48]. B: Binding of TA-Get3 can occur with either ATP or ADP bound. ATP hydrolysis and release of phosphate is required for recycling of the Get3 dimer from the membrane to the cytosol [47]. C: The TA can bind to the open dimer, and ATP stabilizes the binding by inducing the closed state. ATP hydrolysis and engagement of the Get3 receptor are followed by release of ADP with return of Get3 to the open state and integration of the TA in the membrane [50]. D: Binding of the TA occurs to the Get dimer in the absence of bound nucleotide. ATP binds after engagement of the Get3 receptor, and release of the TA depends on ATP hydrolysis [51].

compensates for the loss of interaction between the two Get homologues. Finally, it must be mentioned that putative ribosome and TA binding sites, predicted by the hypothesis that the Get4/5 complex captures tail anchors at the exit of the ribosomal tunnel, have not been identified so far.

3. Insertion into other organelles

3.1. Peroxisomes

Whereas targeting to the ER membrane occurs *via* machinery dedicated to TA proteins, investigations carried out up till now on peroxisomal TA proteins, indicate that they are inserted by a mechanism common to the post-translational insertion of other class I peroxisomal membrane proteins. Class I peroxisomal proteins depend on the import receptor Pex19. Pex19 binds its newly synthesized substrates in the cytosol and, by associating with its membrane integrated receptor, Pex3, delivers them to the peroxisomal membrane ([56], but see also [57]). Halbach et al. [58] identified two Pex19 recognition sequences in the TMD and lumenal sequence of the mammalian TA protein Pex26, and demonstrated that the interaction between these sequences and Pex19 is required for delivery of Pex26 to peroxisomes. In the absence of the lumenal recognition sequence, Pex26 was inappropriately delivered to mitochondria [58]. Similar Pex19 recognition sequences were identified also in yeast Pex15, a TA protein thought to be the functional orthologue of mammalian Pex26 [58]. The interpretation of the data for the yeast protein is however complicated by the finding that its distribution is shifted to mitochondria in Δ□□□□□ yeast cells, suggesting that, as is the case for a large number of yeast peroxisomal membrane proteins [57] it normally reaches peroxisomes by membrane traffic, after insertion into the ER by the Get-dependent pathway [29].

Another TA protein whose targeting to peroxisomes has been studied is the fission protein Fis1. Human Fis1 localizes mainly to mitochondria but a small amount also targets the peroxisomes. As in the case of Pex26, delivery to peroxisomes is Pex19-dependent [59]. The recognition sequence for Pex19 is at the extreme C-terminus and is not required for mitochondrial targeting. It will be interesting to see whether the Pex19-dependence is confirmed for other peroxisomal TA proteins.

3.2. Mitochondria

TA protein delivery to the Outer Mitochondrial Membrane (OMM) appears to depend on physico-chemical features of the tails rather than on defined signals. Whereas the ER targeting machinery can accommodate a large variety of TA lengths, hydrophobicity and flanking charges, TA proteins targeted to the OMM are generally characterized by TMDs of moderate hydrophobicity. In mammals, this moderately hydrophobic TMD is usually flanked on one or both sides by positive charges. Mutagenesis studies on selected OMM proteins carrying these features have demonstrated that both are essential for correct targeting, and altering either one of them causes rerouting of

the investigated TA proteins to the ER [60–65]. As illustrated in a previous review [9], however, although these features on the average clearly distinguish the population of mitochondrially targeted tails from their ER counterparts, and although deletion of these properties by site directed mutation results in loss of correct targeting, there is quite some overlap in hydrophobicity and positive charge between OMM and ER TA proteins. Thus, these features alone cannot explain specific *in vivo* targeting. An additional TMD feature reported to favor mitochondrial targeting is the propensity for α-helix formation [66]. The generality of this interesting observation deserves to be tested on a larger number of artificial and natural sequences with different targeting specificity.

In fungi and plants, the presence of flanking positive charges does not distinguish OMM from ER-targeted TA proteins, and moderate hydrophobicity seems to be the only clear hallmark for OMM targeting [2,4]. This may explain why mammalian OMM TA proteins are not always faithfully targeted in the yeast system (our unpublished results, see also [67]).

Investigations on the nature of the machinery responsible for TA protein targeting to the OMM have yielded conflicting results. Recent work has excluded the participation of the Translocase of the Outer Membrane (TOM) machinery in insertion of three different TA substrates (Bak, BclXL, and OMP25) in semi-intact mammalian cells [68] and of the yeast mitochondrial fission protein Fis1 *in vitro* [65]. On the other hand, TOM20 has been implicated in the import of Bcl-2 [64,69], while TOM22 has been identified as the receptor for the proapoptoic TA protein Bax [70]. In the latter case, the targeting signal is contained in the N-terminal domain of Bax. Similarly, increased targeting of Bcl-2 was reported to be mediated by the interaction of its N-terminal domain with an OMM-localized binding partner (FKB35; [71]). TOM22 itself has been considered a TA protein (although its polar C-terminal domain of 41 residues exceeds the defining length of 30 residues), and found to depend on both TOM20 and TOM70 for its import [72]. Very interestingly, the β-barrel-specific sorting and assembly machinery/topogenesis of β-barrel proteins (SAM/TOB) complex facilitates integration of TOM22 into the OMM [73]. All these results suggest that TA proteins may be targeted to mitochondria by more than one mechanism.

Part of the difficulty in investigating TA targeting to the OMM derives from the moderately hydrophobic nature of the TMD of most mitochondrial TA proteins. This feature predicts that many of them should be able to insert into lipid bilayers without assistance, and, indeed, insertion into protein-free bilayers has been demonstrated for Fis1 [65], for a mitochondrially targeted isoform of b5 (our unpublished results), and for Bcl-2 [19]. This unassisted insertion *in vitro* may mask specific, chaperone-mediated targeting occurring *in vivo*. An example of this problem is given by our studies on the targeting of the two b5 isoforms, which are specifically localized to the OMM and the ER [74]. Mutation of the C-terminal polar domain of the ER targeted protein to contain a net positive charge results in a protein (b5-RR) that is specifically targeted to mitochondria *in vivo*. Yet, *in vitro*, this mutant inserts into ER microsomes as efficiently as its wild-type counterpart [62]. Mitochondria added to the translocation reaction compete with ER microsomes, and sequester the TA substrate to the OMM. However, this competition is as efficient for the mitochondrial as for the ER-targeted substrate (our unpublished results), illustrating how the specific *in vivo* targeting of the two b5 isoforms is not recapitulated *in vitro*.

3.3. Plastids

TA protein targeting to plastids has been investigated for a Chloroplast Outer Envelope (COE) isoform of cyt b5 [75], for the GTPase import receptors Toc33 and 34 [76,77] and for a COE protein (OEP9) of unknown function [77].

The Arabidopsis genome contains five putative cyt b5 isoforms, all with a net positive charge at the C-terminus [12]. One of these isoforms (AtCb5-3) is sorted to the ER and another (AtCb5-6) to the COE [75]. In cells lacking chloroplasts, the latter is targeted to the OMM [75], illustrating once again that mitochondria represent a common destination for TA proteins when their normal targeting pathway is nonfunctional. Not clarified is the basis for the COE isoform's preference for the COE under normal conditions, nor for the discrimination between the ER and the COE of the two investigated isoforms. The TMDs of both have similar flanking positive charges, however, hydrophobicity analysis reveals that the ER-targeted TMD is slightly more hydrophobic than its COE-targeted counterpart. Whether this difference explains the specific targeting of the two isoforms remains to be established.

More mechanistic insight was obtained from the investigations on the other three COE proteins [76,77], which revealed that OEM9 and Toc 33/34 partially differ in their targeting mechanism. Targeting determinants of OEM9 are, as is generally true for TA proteins, entirely contained in the tail region. In contrast, Toc33 and 34 targeting depends also on information within the N-terminal GTPase domain. Insertion of both proteins into chloroplasts was enhanced by proteins exposed on the surface of the COE, however, only Toc33 and 34 insertion was facilitated by their own presence in the membrane. Furthermore, the effect of lipid composition on the association of Toc33/34 and OEM9 to protein-free liposomes was different. Very interestingly, however, both OEM9 and Toc33/34 interact with Arabidopsis Ankyrin Repeat Protein-2A (AKR2A), a chaperone recently demonstrated to be involved in targeting of COE proteins [78]. Thus, as is the case for peroxisomes, targeting of TA proteins to plastids may depend on chaperones involved in the post-translational insertion of other, non-TA, COE proteins.

4. The targeting problem: A fierce competition between chaperones

The demonstration that some TA proteins can translocate their C-terminus across protein-free bilayers [16,20,65,76] suggests that also those which require chaperone-mediated assistance may in the final step spontaneously integrate into the bilayer. For this reason, we believe that specific targeting, rather than translocation, constitutes the major, incompletely solved problem in the field of TA protein biogenesis.

Membranes of the secretory pathway downstream to the ER are nonpermissive for TA protein insertion. This nonpermissivity is at least in part due to lipid composition, notably, to sterol content [16,65]. Therefore, in animal and fungal cells, there are only three possible target membranes for newly synthesized TA proteins: the ER, peroxisomes and the OMM. A fourth target, the plastid outer envelope, is present in plant cells.

The discovery of the Get3/TRC40 pathway has represented a major breakthrough for our understanding of TA protein targeting. The presence of a specific Get3 receptor (Get1/2) on the ER membrane nicely accounts for Get3-mediated targeting to the ER, and it is presumed that a functional homologue on the ER of higher eukaryotes plays the same role. Nevertheless, the specific targeting to the ER of TA proteins that are able to insert in the absence of TRC40/Get3, such as b5 and PTB1B [18,20], remains mysterious. It is possible that *in vivo* these proteins do associate with Get3/TRC40, as suggested by the formation of a stable complex between b5 and TRC40 when the two proteins are expressed together in *E. coli* [53]. Another possibility is that yet other unidentified chaperones are involved, as suggested by our recent work [18]. Whatever the targeting factors are, they must in the case of the unassisted TA proteins have a dual role, preventing inappropriate spontaneous insertion into all permissive bilayers while favouring delivery to the correct target.

TA proteins targeted to peroxisomes, the OMM, and the COE must avoid binding Get3/TRC40. On the one hand, they may have low affinity for this chaperone, as suggested by the observation that the composite hydrophobic groove of Get3 is of sufficient length to host longer TMDs than those normally present in TA proteins targeted to organelles other than the ER. Furthermore, the extremities of the groove are rich in basic

residues, which could discourage binding of TMDs flanked by positive charges, as found in mammalian OMM TA proteins [48]. On the other hand, they may have stronger affinity for other targeting factors which deliver them to the appropriate destination. This appears to be the case for the peroxisomal TA proteins that depend on PEX19 [58,59], and it may turn out that AKR2A has a similar function for plastid-targeted TA proteins [77].

This leaves out the OMM, for which the targeting factors still remain mysterious. It might be speculated that targeting to the ER, to peroxisomes and to plastids is signal-mediated, and that the OMM represents the default pathway for TA protein insertion, i.e., any TA protein without targeting information for other organelles will insert into the OMM. We find this hypothesis unlikely, because (i) in the absence of the Get pathway in *Saccharomyces* not all ER TA proteins are mislocalized to mitochondria [29], suggesting that features additional to lack of Get3 binding are required for mitochondrial targeting; and (ii) *in vitro* at least some OMM proteins are capable of integrating into ER microsomes [62,65], hence *in vivo* these proteins must interact with factors that hinder their inappropriate insertion into the ER. While these factors await identification, we speculate that Hsc70/Hsp40 may play a role in mitochondrial targeting. *In vitro*, in the complete RRL system, these chaperones interact with ER-targeted TA proteins with moderately hydrophobic TMDs (b5, PTB-1B, and Bcl-2), and not with classical TRC40 substrates [27]. It could be that *in vivo* the Hsc70 system is out-competed by TRC40 in the case of ER-targeted TA proteins, even of those with weakly hydrophobic TMDs, but that it does win in the competition for mitochondrial TA proteins. This hypothesis is attractive because the OMM import receptor TOM70 has been recognized as a binding partner of Hsp70. The Hsp70/TOM70 interaction functions in the delivery of a set of mitochondrial precursor proteins to the mitochondrion for subsequent import [79].

Other specificity factors may contribute to the fate of newly synthesized TA proteins. For instance, in addition to the effects of sterols, additional differences in lipid composition of the target membranes could influence targeting, a point that deserves further investigation. Additional possibilities that might be considered are pre-translational targeting phenomena, i.e., localization of mRNA's coding for differently localized TA proteins in close proximity to the target organelle, or post-insertion phenomena, i.e., initial indiscriminate insertion of a TA protein into all permissive membranes, followed by its rapid degradation in the inappropriate organelle. At least for b5, we can exclude these two mechanisms, because: first, microinjected b5 *protein* is correctly targeted to the ER in cultured cells [18], indicating that the targeting information is in the protein and not in the mRNA; second, in *S. cerevisiae*, b5 carrying an epitope with a consensus for N-glycosylation, is completely glycosylated after a pulse of only 5 min, indicating that it is all in the ER [20]. It is unlikely that in this short time span, the protein could be inserted into other membranes and then be completely degraded.

Because of the above considerations, we favor the idea that competition between chaperones, possibly in conjunction with differences in bilayer lipid composition, underlies the complexities of TA protein targeting. As schematized in Fig. 3, upon release from the ribosome, TA proteins presumably encounter a number of potential chaperones. Different *on* and *off* rates of the interactions may determine which chaperone system, and hence which target membrane, wins out in the competition. Subtle sequence differences and changes in the availability of different chaperone systems may alter the outcome of the competition, resulting in some cases in dual targeting (Table 1), and explaining why some ER TA proteins are re-directed to the OMM in the absence of the Get system. This non-univocal mode of targeting may be exquisitely amenable to regulation, and we can expect examples of this to be discovered in the future.

5. Conclusions

The increased interest in TA proteins and the development of appropriate assays to investigate their membrane integration has led to great progress in our understanding of the biogenesis of this class of proteins. Notably, the discovery of a novel chaperone system (Get3/TRC40) has generated a lot of excitement. TA protein biogenesis has

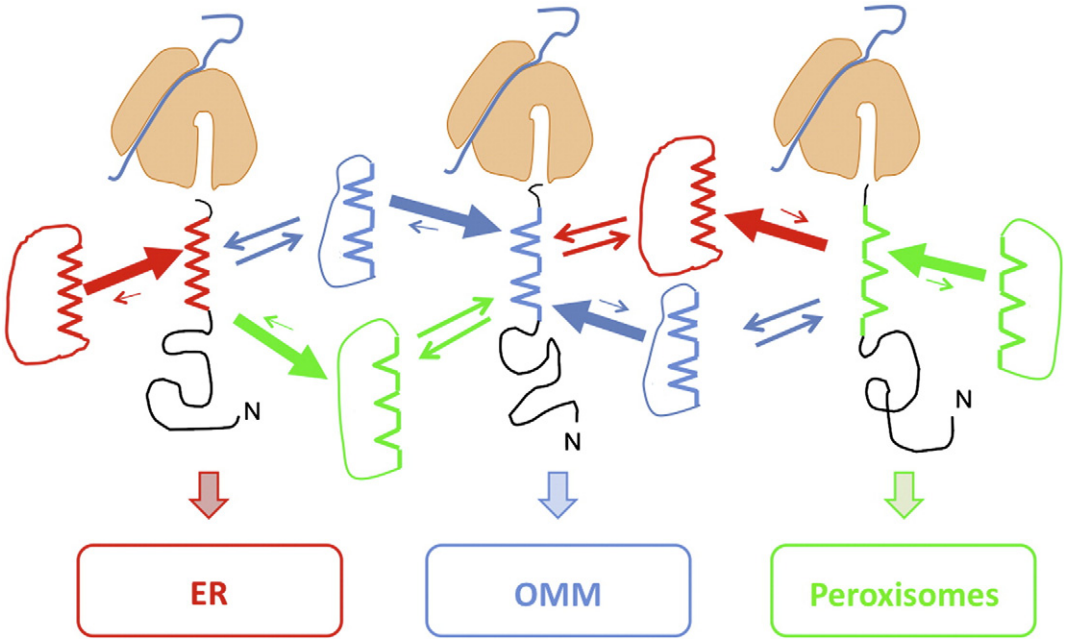

Fig. 3. Simplified scheme illustrating how competition between chaperones may govern TA protein targeting. The cartoon illustrates how different TA proteins can potentially interact with many different chaperones. Chaperones that mediate insertion into the ER, OMM and peroxisomal membrane are in red, blue, and green respectively. The colored arrows pointing away or towards each chaperone represent k_{on} (upper arrow) and k_{off} (lower arrow) rate constants of that chaperone for a given TA substrate. The thickness and length of each arrow is proportional to the postulated rate constants. The most rapidly and stably binding chaperone will determine the TA substrate's destination, but under altered conditions (e.g., depletion of one chaperone system), alternative fates for a given TA protein are possible. The cartoon is not meant to illustrate the exact mode of action of any particular chaperone system, but is presented only as a general framework. The choice of the relative *on* and *off* rates has been made in consideration of the observation that TA proteins doubly localized to ER and OMM, as well as to OMM and peroxisomes have been described (Table 1), but none are known to be shared by the ER and peroxisomes.

proven to present more intricacies that had perhaps been anticipated. Thus, notwithstanding the recent progress, we still have a lot to learn, and can anticipate new unexpected developments during the next few years.

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