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A structural perspective on tail-anchored protein biogenesis by the GET pathway

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Many tail-anchored (TA) membrane proteins are targeted to and inserted into the endoplasmic reticulum (ER) by the 'guided entry of tail-anchored proteins' (GET) pathway. This post-translational pathway uses transmembrane-domain selective cytosolic chaperones for targeting, and a dedicated membrane protein complex for insertion. The past decade has seen rapid progress towards defining the molecular basis of TA protein biogenesis by the GET pathway. Here we review the mechanisms underlying each step of the pathway, emphasizing recent structural work and highlighting key questions that await future studies.

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Introduction

Eukaryotic membrane proteins of the plasma membrane and compartments of the secretory and endocytic pathways are first inserted into the endoplasmic reticulum (ER) membrane. The majority of these are inserted by a universally conserved co-translational pathway mediated by the signal recognition particle (SRP), its membrane receptor, and the Sec61 protein translocation channel [1,2]. However, a functionally diverse set of eukaryotic membrane proteins known as 'tail-anchored' (TA) proteins cannot access this pathway [3]. The eukaryotic genome encodes hundreds of TA proteins, each with a cytosolic-facing N-terminal domain and a single C-terminal transmembrane domain (TMD) that serves both as a membrane anchor and as a targeting signal [4–7]. Because the TMD remains sequestered inside the ribosome exit channel until after translation is complete, co-translational targeting and insertion is precluded.

Work over the past decade has identified multiple pathways for post-translational TA protein biogenesis at the ER. The 'guided entry of tail-anchored proteins' (GET) pathway targets TA protein clients containing highly hydrophobic TMDs [8,9], while the 'ER membrane complex' (EMC) [10] exhibits a preference for TA proteins with lower hydrophobicity TMDs [11]. Additionally, the recently described SND pathway may target TA proteins that fail to engage the GET or EMC pathways [12]. These pathways appear to operate in parallel to mediate TA biogenesis at the ER.

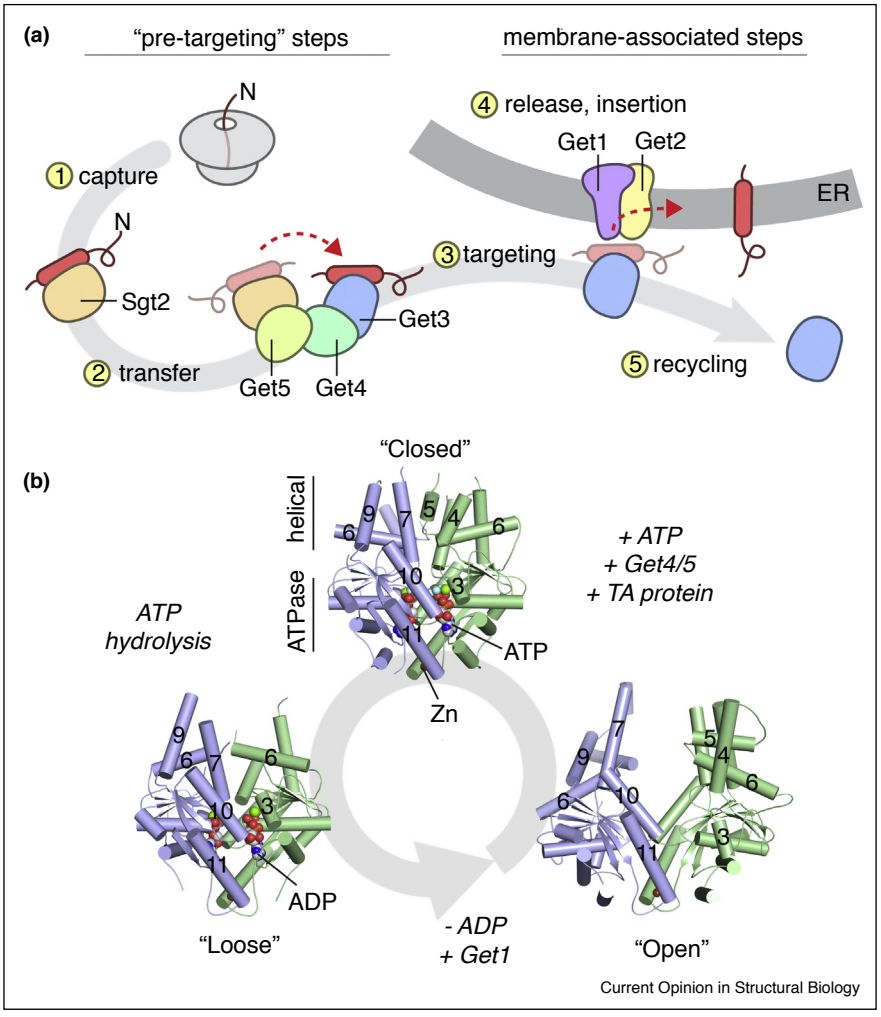
The best characterized of these pathways is the GET pathway. Since its discovery, biochemical, genetic and structural studies have defined the components and molecular logic for much of the pathway. A simplified model for TA protein biogenesis by the yeast GET pathway is shown in Figure 1a. The pathway begins when the cytosol chaperone Sgt2 captures newly synthesized TA proteins via their hydrophobic TMDs, at or near the ribosome. Next, the Get4/5 'scaffolding complex' recruits the Sgt2–TA complex via Get5, while Get4 recruits the central targeting factor, Get3. A hand-off reaction within this 'pre-targeting' complex results in transfer of sufficiently hydrophobic TA proteins from Sgt2 to Get3. TA-loaded Get3 then dissociates from Get4, and this 'targeting complex' is directed to the ER by an interaction with the Get1/2 complex. At the membrane, Get1/2 disrupts the TA protein binding site in Get3, releasing the TA protein for insertion into the membrane. Finally, Get3 is recycled to the cytosol to initiate a new round of targeting.

In this review we summarize our current understanding of the GET pathway, with an emphasis on the structural mechanisms underlying each step in the pathway. The focus here is on the yeast system — where much of the structural work has been done to date — but mammalian-specific features of the pathway are also discussed. In addition, we highlight key questions that await future structural and biochemical studies.

The Get3 conformational switch

Get3 plays a central role in the GET pathway by binding clients in the cytosol, shielding them through the crowded aqueous environment, and releasing them at the ER membrane for insertion. The Get3 fold comprises a helical subdomain that is structurally and functionally coupled to a core ATPase domain (Figure 1b). Get3 functions as a homodimer in which two subunits are linked by a tightly coordinated zinc ion. Crystallographic,

Figure 1



Overview of the GET pathway. **(a)** A simplified model for TA protein biogenesis by the yeast GET pathway. See text for details. **(b)** The Get3 conformational cycle. The homodimeric Get3 ATPase (blue, green) adopts different conformations in response to ligand binding. These conformational transitions occur about a hinge point centered on a zinc ion (brown sphere) bound within the nucleotide-binding domain, located at the dimer interface. These range from 'closed' conformations with an extensive dimer interface that spans each subunit [PDB ID 2woj], to 'loose' conformations with an increasingly disrupted dimer interface (starting from the helical subdomains) [PDB ID 3iqx], to 'open' states in which the two subunits are splayed apart [PDB ID 3h84]. Binding to ATP-, Get4/5 and/or TA protein drives Get3 towards more 'closed' conformations, while ATP hydrolysis, Get1 binding and/or nucleotide dissociation drives Get3 towards more 'open' conformations.

computational and biophysical studies reveal different conformations for Get3 [13–18], ranging from: (1) 'closed' conformations with an extensive dimer interface that extends along the entire length of each monomer and harbors the TMD binding site; (2) intermediate, 'loose' conformations in which the dimer interface becomes progressively disrupted, beginning with the helical subdomains; and (3) 'open' conformations in which the two Get3 subunits are splayed apart. As detailed below, these conformations are regulated by interactions with different ligands — nucleotides, the Get4/5 scaffolding complex, TA protein clients and the ER-localized insertion machinery, Get1/2 — to coordinate cycles of TA protein binding and release.

Events in the cytosol

TA protein capture by Sgt2

The cytosolic chaperone Sgt2 (SGTA in humans) is considered the most upstream factor in the GET pathway [19,20•]. Sgt2 captures newly synthesized TA proteins that are destined for the ER, but precisely where and when this occurs is unclear (Figure 2a). The yeast Get4/5 complex has been proposed to associate with ribosomes [21,22], and the analogous complex in mammals (the TRC35–UBL4A–Bag6 complex) is recruited to ribosomes harboring a TMD inside the exit channel [23]. By localizing Sgt2/SGTA to the surface of the ribosome, these interactions might facilitate the rapid capture of TA proteins, thereby minimizing opportunities for

aggregation. However, the location of the putative ribosome binding site, and the molecular mechanism underlying recruitment to the ribosome remain to be explored.

Sgt2 functions as a homodimer, and comprises three different structural domains connected by flexible linkers (Figure 2a): (1) an N-terminal dimerization domain, (2) a central tetratricopeptide repeat (TPR) domain that mediates binding to various cytosolic chaperones, and (3) a small C-terminal domain implicated in TMD binding [19,24,25]. Structures are known for the first two domains (Figure 2b) [24,26], but not the C-terminal domain. Consistent with its role in TMD binding [19], the Sgt2 C-terminal domain is flexible, rich in methionine residues, and predicted to be largely helical. Defining how these elements contribute to TMD capture is an important future goal.

TA protein transfer to Get3

Following capture by Sgt2, TA protein transfer requires the heterotetrameric Get4/5 complex, which functions as a scaffold onto which Sgt2–TA and ATP-bound Get3 assemble [19,27••,28•]. Three functions of the 'pre-targeting' complex appear to be critical for this process. First, Get4/5 preferentially binds to ATP-bound (closed) Get3, which harbors the TMD binding site (see below), priming it for TA transfer [29]. Second, Get4/5 binding inhibits the ATPase activity of Get3 [30]. Third, Get4/5 brings Sgt2–TA and Get3-ATP into close proximity, facilitating the protected transfer of TA substrate [20•].

Structures of individual components and subcomplexes have started to provide a framework for understanding TA protein transfer (Figure 2b,c). Get5 is a multi-domain protein comprising an N-terminal region that binds tightly to Get4, a central ubiquitin-like (UBL) domain that recruits Sgt2, and a C-terminal dimerization domain [31]. Get4 adopts an α-solenoid fold [29,32,33]; the C-terminal end of Get4 forms the binding site for Get5, while the N-terminal end mediates high-affinity binding to Get3.

A low resolution crystal structure of ATP-bound Get3 in complex with Get4 and a monomeric N-terminal peptide of Get5, showed that Get4 binds across the Get3 dimer interface (Figure 2c) [34••]. This orientation is only compatible with closed Get3 conformations, providing an elegant structural explanation for selective binding of Get4 to ATP-bound Get3. Intriguingly, a portion of Get4 binds near the Get3 active site, and mutational analysis shows that residues within this interface are critical for inhibition of the Get3 ATPase activity [34••]. The structural basis of this inhibitory interaction awaits a high-resolution structure of the ATP-bound Get3–Get4 complex.

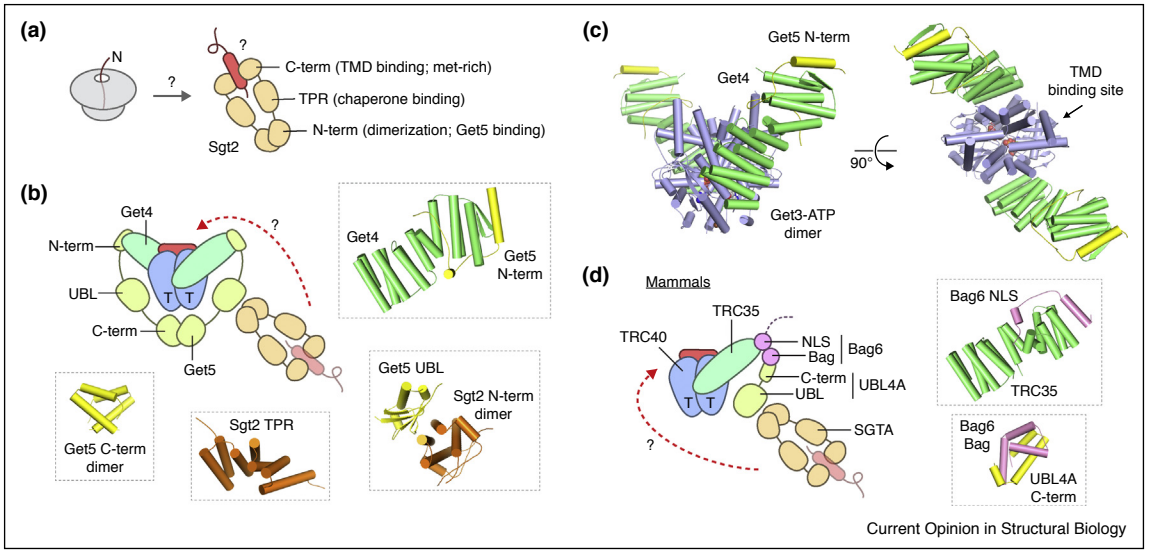
In the absence of a structure of the fully assembled complex, how Get4/5 organizes Sgt2 and Get3 for TA protein transfer remains unclear. By analogy to the 'hydrophobic handoff' of cholesterol from NPC2 to NPC1 during exit from lysosomes [35,36], an attractive model is that the Get4/5 scaffolding complex brings the TMD binding sites of Sgt2 and Get3 into direct contact to allow for protected transfer of their highly hydrophobic cargo. Solution studies indicate that the full-length Get3/4/5 complex contains two copies of each protein — i.e., one Get3 dimer bound to a Get4/5 heterotetramer [27••,37]. This suggests a simple model in which two copies of Get4 bind simultaneously to opposite sides of the symmetric Get3 dimer (Figure 2b,c). Consistent with this, full-length, heterotetrameric Get4/5 complexes bind ~40-fold more tightly to ATP-bound Get3 than truncated, heterodimeric Get4/5 complexes [38], presumably reflecting a strong avidity component to the interaction. However, alternative models are also possible, including an asymmetric arrangement in which only one copy of Get4 binds to Get3 in the complex [37]. Structural analysis of ATP-bound Get3 in complex with full-length Get4/5 is needed to clarify the overall architecture of the Get3/4/5 complex.

Sgt2 is recruited to Get4/5 via a direct interaction of its N-terminal dimerization domain with the UBL domain of Get5 (Figure 2b). Intriguingly, solution studies suggest that Sgt2 binding to one Get5 subunit prevents binding of a second Sgt2 to the other Get5 subunit [24]. Structural studies of the fully assembled pre-targeting complex are needed to define the molecular basis of this asymmetry, and the relative orientation of Sgt2 and Get3 prior to TA transfer.

Once the TA protein is transferred to Get3, the Get3–TA complex dissociates from Get4/5. Biochemical studies indicate that TA binding and nucleotide hydrolysis (possibly stimulated by TA protein binding) weaken the affinity of Get3 for Get4/5 [38]. The conformational changes underlying this process remain obscure, but likely involve remodeling of the Get3 dimer interface from a closed conformation towards a more intermediate ('loose') conformation (Figure 1b) [39].

TA transfer in the mammalian system involves a three protein scaffolding complex comprising TRC35 (Get4 homolog) and UBL4A (Get5 homolog) and a unique subunit, Bag6 [23]. In contrast with the yeast components, TRC35 and UBL4A (which is a monomeric protein) do not directly interact; instead, TRC35 and UBL4A bind to short motifs at the C-terminal end of Bag6 (Figure 2d). The structural details of the scaffold are different [40••,41••], but result in what is likely to be an analogous arrangement of TRC40 (Get3 homolog) and SGTA (Sgt2 homolog) in the fully assembled pre-targeting complex. A TA protein that fails to transfer onto

Figure 2



Pre-targeting steps. **(a)** Newly synthesized TA proteins are captured at or near the ribosome by Sgt2, a homodimeric protein comprising three domains: an N-terminal dimerization domain, a central TPR domain, and a C-terminal TMD-binding domain. Structures are known for the N-terminal dimerization domain [PDB ID 2lxc; see (b)] and central TPR domain [PDB ID 3sz7; see (b)]. How the methionine-rich, helical C-terminal domain binds to TA proteins remains unclear. **(b)** A plausible model for transfer of a TA protein from Sgt2 to Get3 via the Get4/5 scaffolding complex. Structures are known for the C-terminal dimerization domain of Get5 [PDB ID 3vej], for the Get5 UBL domain bound to the Sgt2 N-terminal dimerization domain [PDB ID 2lxc], and for Get4 bound to the N-terminal extension of Get5 [PDB ID 3lku]. Biochemical analysis suggests that the transfer complex accommodates only one Sgt2 dimer per complex, despite the presence of two binding sites (Get5 UBL domains); the structural basis for this asymmetry remains unclear. **(c)** Low resolution crystal structure of an ATP-bound Get3 dimer bound to two copies of the Get4–Get5N complex [PDB ID 4pwx]. **(d)** Model of the mammalian Bag6 complex, colored as in (b). In contrast with the yeast complex, where Get4 and Get5 interact directly, the mammalian homologs TRC35 (Get4) and UBL4A (Get5) assemble on the NLS [PDB ID 6au8] and Bag domain [PDB ID 4wwr] respectively, of the mammalian-specific subunit, Bag6. In turn, the mammalian homologs TRC40 (Get3) and SGTA (Sgt2) are recruited to the scaffold by TRC35 and the UBL domain of UBL4A, respectively.

TRC40 instead engages a quality control module within the Bag6 subunit, effectively committing it to proteasomal degradation [20•]. The structural basis of TMD binding to Bag6 is not known.

The Get3–TA protein targeting complex

Crystal structures of Get3–TA–ATP complexes define a canonical binding mode in which the client TMD binds within the composite hydrophobic groove of closed Get3 (Figure 3) [27••]. This helical, methionine-rich groove is dynamic, as evidenced by higher B-factors in both the presence and absence of a bound TMD. Although disordered in the structure, a conserved motif ('TRC40-insert') including helix 8, appears to function as a dynamic lid, shielding the TMD through the cytosol while still allowing substrate release at the membrane (Figure 3b).

The bound TMD buries nearly 1500 $Å^2$ of hydrophobic surface area, distributed nearly evenly between the two Get3 subunits (Figure 3c). This represents ~50% of the ordered hydrophobic surface area in the groove and is significantly greater than in the SRP54–signal peptide interaction [42,43]. The availability of such a large surface area likely explains the preference of Get3 for highly hydrophobic TMDs [11,19,28•], and its ability to accommodate sequences of differing lengths and amino acid composition [6].

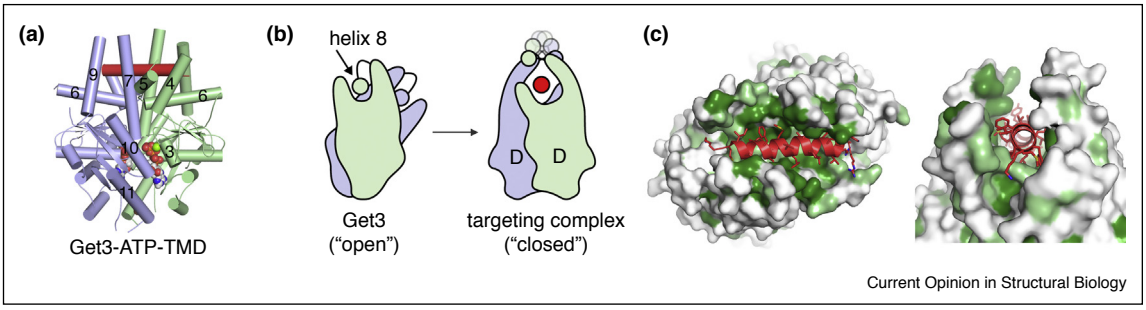
ATP hydrolysis by the Get3–TA complex is necessary before the targeting complex can release its cargo at the membrane [44–46]. TA binding has been proposed to stimulate the ATPase activity of Get3 [30], but precisely when this occurs remains unclear. Importantly, TA binding to Get3 dramatically slows the rate of ADP dissociation and ATP rebinding [30]. This presumably occurs because the TMD pins together the two Get3 subunits such that the nucleotide binding sites remain protected from the cytosol, even as Get3 begins to transition to 'loose' conformations following hydrolysis. This provides a window of time in which the Get3–TA targeting complex can productively engage with the Get1/2 machinery for TA protein release and insertion.

Events at the membrane

Membrane targeting

After a TA protein is loaded onto Get3, the complex is targeted to the Get1/2 transmembrane complex at the ER [9,44–46]. Although early models proposed that Get1/2

Figure 3



The Get3–TA protein targeting complex. **(a)** Crystal structure of a 'closed' Get3 dimer (blue, green) bound to ATP (spheres) and the TMD of a tail-anchored protein, Pep12 (red) [PDB ID 4xtr]. The client TMD lies across the Get3 dimer interface. The distal ends of Get3 helices 4, 5, 7 and 9, and all of helix 8, are disordered in the presence of a bound TMD, underscoring the intrinsic flexibility of the binding site. **(b)** Helix 8 is part of a conserved sequence motif ('TRC40-insert') in the Get3 family of SIMIBI NTPases. This helix protects the disrupted hydrophobic groove of Get3 in the absence of TA protein (left), and dynamically shields the bound TA protein TMD in the targeting complex (right). **(c)** Surface representations of the TMD binding site, colored from least (white) to most (green) hydrophobic. The large hydrophobic surface area presented by the Get3 composite groove is consistent with the preference of the GET pathway for TA proteins with extremely hydrophobic TMDs.

might function as a heterotetramer [44,45], it is now clear that a Get1/2 heterodimer, comprising a single copy of each subunit, is both necessary and sufficient for TA targeting and insertion [47]. Get1 and Get2 (WRB and CAML in humans) [48–50] each contain three predicted TMDs through which the two subunits associate, and large cytosolic-facing domains containing conserved residues that mediate binding to Get3.

The targeting complex, likely in an ATP-bound or ADP-bound state (depending on the timing of hydrolysis), is first captured at the membrane by Get2. Solution studies indicate that Get2 binding is insensitive to the conformational state and ligand occupancy of Get3 [38,44–46]. Consistent with this, crystal structures of ATP-bound and ADP-bound Get3 in complex with a portion of the long, flexible cytosolic region of Get2 reveal contacts that are largely restricted to one subunit of a closed Get3 dimer (the α10/α11 region) (Figure 4a) [44,45]. Notably, this surface of Get3 does not change conformation in response to nucleotide or TA protein binding, rationalizing the observed conformation-independent binding of Get2.

TA protein release

In contrast to Get2, Get1 binding is sensitive to the conformational state and ligand occupancy of Get3 [38,44–46]. Fully closed conformations (e.g., Get3–TA–ATP) do not bind with high affinity to Get1. However, in the presence of Get2, Get1 is able to interact with loose, ADP-bound states of Get3 [47]. The dynamic nature of this conformation leads to formation of a tight complex between Get1 and nucleotide-free Get3 that drives TA protein release [38,39,44–46].

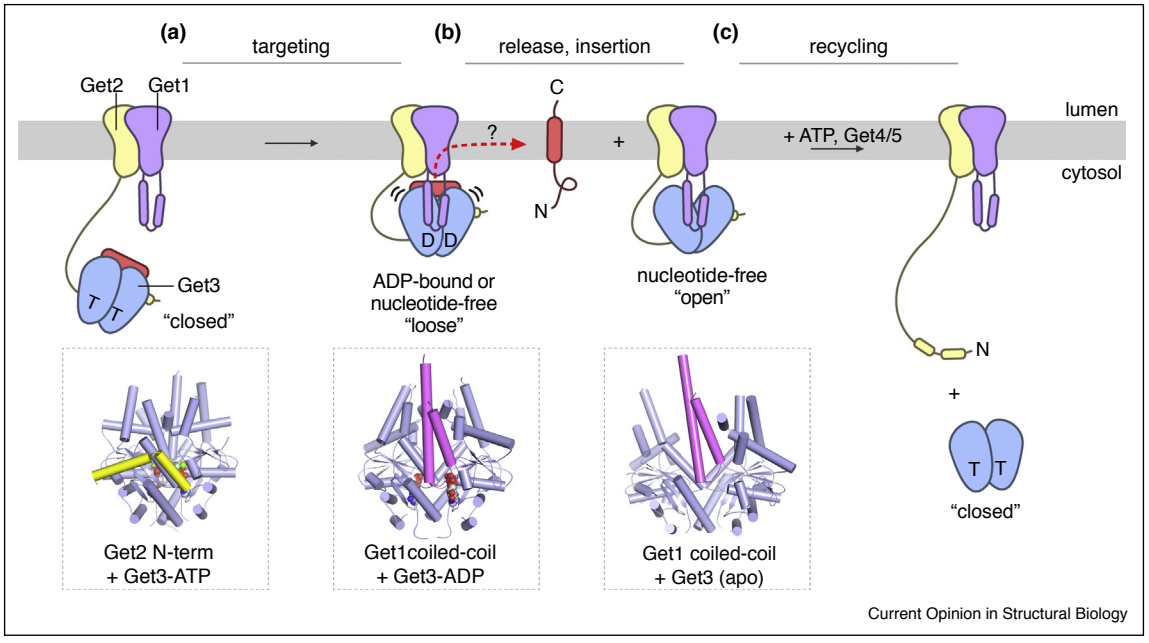
Structural insight for this comes from crystal structures of ADP-bound and nucleotide-free Get3 complexes with the cytosolic coiled-coil motif of Get1 [44,45,51]. In the ADP-bound complex (Figure 4b), the Get3 dimer adopts a loose conformation in which contacts are primarily to one subunit of Get3 (via the α10/α11 region) and the ATP binding site is unperturbed. This presumably weak interaction is likely facilitated by Get2 binding on the opposite side of Get3 [47]. In the nucleotide-free Get3 complex, the Get1 coiled-coil wedges between the two Get3 subunits, such that Get3 adopts an open conformation (Figure 4c). In this conformation, the Get1 coiled-coil makes extensive contact with both subunits, including the conserved ATPase motifs of the active site, which now becomes solvent exposed. Importantly, this high-affinity interaction completely disrupts the composite TA binding site in Get3, driving substrate release.

TA protein insertion

The mechanism of TMD insertion remains unclear, but it appears to involve the TMDs of Get1 and Get2. The rigid interaction between the Get1 coiled-coil and Get3 suggests that the substrate TMD is released parallel to the bilayer surface, in close proximity to the transmembrane domains of Get1/2 (Figure 4b). Mutations within the Get1/2 TMDs impair insertion, and crosslinking analysis demonstrates direct contacts between the released TA protein and the Get1/2 TMDs [52•]. Thus, Get1/2 functions as a bona fide TA protein insertase that recognizes TMDs and provides a path from the cytosol into the bilayer.

Although the structural basis of insertion remains unclear, insight comes from the recent discovery that Get1 is a member of the 'Oxa1 superfamily', whose members include the bacterial insertase YidC, a family of archaeal DUF106 proteins, the EMC3 subunit of the TA protein insertase EMC, and an ER-resident protein called

Figure 4



Membrane-associated steps. The heterodimeric Get1/2 complex coordinates events at the ER membrane. **(a)** Targeting. A conserved helical motif at the end of a long, flexible cytosolic N-terminus of Get2 (yellow) initially binds to the targeting complex, likely in an ATP-bound or ADP-bound state. A crystal structure of the Get2 N-terminus bound to 'closed', ATP-bound Get3, is shown [PDB ID 3zs9]. Contacts are mainly to a single Get3 subunit, and are insensitive to the conformation of the Get3 dimer. **(b)** Following hydrolysis, the dimer interface within the targeting complex becomes increasingly 'loose' (Figure 1b), and the conserved, cytosolic coiled-coil motif of Get1 makes initial contact with Get3. A crystal structure of the Get1 coiled-coil bound to 'loose', ADP-bound Get3, is shown [PDB ID 3vlc]. At this stage, most of the contacts are to a single subunit of Get3, and the nucleotide binding site remains protected from Get1 and bulk solvent. Subsequently, the dynamic nature of the post-hydrolysis targeting complex allows Get1 to wedge apart the Get3 dimer. A crystal structure of the Get1 coiled-coil bound to nucleotide-free, 'open' Get3 is shown [PDB ID 3zs8]. At this stage, the Get1 coiled-coil makes contacts to both subunits of Get3, and inserts the tip of its coiled-coil into the nucleotide binding site. In this high affinity complex, the composite hydrophobic groove of Get3 is disrupted, driving release of the TA protein for insertion into the bilayer through the Get1/2 complex. **(c)** Recycling. ATP- and Get4/5 binding recycles Get3 to the cytosol to initiate a new round of targeting.

TMCO1 [53•]. Crystal structures of YidC and an archaeal family member (Ylp1) reveal a shared structural core comprising three TMDs that harbor a lipid-exposed hydrophilic groove that can be crosslinked to nascent TMD-containing substrates [54,55]. Identification of an ancient evolutionary relationship between Get1 and YidC suggests that they share similarities in structure and mechanism for TMD insertion. Confirmation of this awaits high-resolution structural analysis of Get1/2/3 complexes.

Get3 recycling

After the TA substrate has been released, Get3 must dissociate from Get1/2, recycling it to the cytosol and vacating Get1/2 for the next targeting complex (Figure 4c). In the high-affinity complex between Get1 and nucleotide-free Get3, the tip of the Get1 coiled-coil overlaps with the ATP binding site of Get3. Consistent with this observation, solution studies show that ATP binding displaces the cytosolic coiled-coil of Get1 from Get3 [38,44,45,51]. The Get4/5 complex may also facilitate release of Get3 from the membrane [38] by sequestering the recycled Get3–ATP complex and priming it for another round of substrate loading.

Outlook

Despite rapid progress in defining the molecular details of TA protein biogenesis by the GET pathway, important questions remain. The structural basis of TA protein capture at the ribosome, and of TMD binding by the C-terminal domains of Sgt2/SGTA, remains unclear. The structural mechanism underlying Get4 inhibition of the Get3 ATPase prior to TA transfer, and how the pre-targeting complex organizes Sgt2 and Get3 for TA transfer remain unknown. Similarly, the conformational changes in Get3 that drive dissociation of the Get3–TA targeting complex from Get4/5 are unclear. Finally, the structure of full-length Get1/2 and the mechanism of TMD insertion into the bilayer remain to be defined. Answers to these questions will require more sophisticated structural approaches including single-particle cryo-EM, which promises high-resolution information for the large and flexible GET pathway complexes that have so far eluded structural analysis.

Acknowledgement

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- • of special interest
- •• of outstanding interest

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