Guiding tail-anchored membrane proteins to the endoplasmic reticulum in a chaperone cascade

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Newly synthesized integral membrane proteins must traverse the aqueous cytosolic environment before arrival at their membrane destination and are prone to aggregation, misfolding, and mislocalization during this process. The biogenesis of integral membrane proteins therefore poses acute challenges to protein homeostasis within a cell and requires the action of effective molecular chaperones. Chaperones that mediate membrane protein targeting not only need to protect the nascent transmembrane domains from improper exposure in the cytosol, but also need to accurately select client proteins and actively guide their clients to the appropriate target membrane. The mechanisms by which cellular chaperones work together to coordinate this complex process are only beginning to be delineated. Here, we summarize recent advances in studies of the tail-anchored membrane protein targeting pathway, which revealed a network of chaperones, cochaperones, and targeting factors that together drive and regulate this essential process. This pathway is emerging as an excellent model system to decipher the mechanism by which molecular chaperones overcome the multiple challenges during post-translational membrane protein biogenesis and to gain insights into the functional organization of multicomponent chaperone networks.

Generation and maintenance of a functional proteome requires the proper folding, assembly, and localization of all of the cellular proteins. Integral membrane proteins comprise over 30% of the proteins encoded by the genome and mediate numerous essential cellular processes, including molecular transport, energy generation, signaling, and cell-to-cell communication. Compared with soluble proteins, the biogenesis of integral membrane proteins poses particularly acute challenges to protein homeostasis in the cell. Before arrival at the appropriate membrane destination, newly synthesized membrane proteins must traverse the cytosol and, in some cases, multiple other aqueous cellular compartments where improper exposure of their transmembrane domains (TMDs) will lead to rapid and irreversible aggregation. In addition, the degeneracy of TMD-lipid interactions poses challenges to the fidelity of their insertion at the appropriate biological membrane, especially in eukaryotic cells that contain multiple membrane-enclosed organelles. The proper localization and folding of membrane proteins therefore relies critically on molecular chaperones, which not only protect nascent membrane proteins from off-pathway interactions but also actively guide them to the correct biological membrane. The mechanism by which the cellular chaperone network overcomes these challenges during membrane protein biogenesis remains an outstanding question.

In the past decade, an increasing number of factors have been described that represent components of multiple, distinct protein-targeting pathways that deliver nascent membrane proteins to diverse organelles such as the endoplasmic reticulum (ER), mitochondria, and peroxisomes (1–6). One of these pathways, the guided entry of tail-anchored protein (GET) pathway, has been studied in exquisite mechanistic detail. This review will summarize recent advances in our understanding of the GET pathway, with a focus on a hierarchical chaperone network found in this pathway. These findings suggest sophisticated solutions to the challenges of membrane protein biogenesis as well as new questions about the role and mechanisms of molecular chaperones during this process.

Diverse targeting pathways accommodate membrane proteins with distinct TMD locations

Diverse pathways mediate the targeting of nascent membrane proteins to the ER, via which proteins enter the endomembrane system in eukaryotic cells. Despite being overly simplistic, it has been useful to conceptualize the multitude of targeting mechanisms in terms of the needs of membrane proteins with distinct TMD locations. For example, most membrane proteins harboring a TMD near the N terminus are recognized by the universally conserved signal recognition particle (SRP) as soon as its first TMD emerges from the exit tunnel of the translating ribosome. Ribosome profiling work in yeast further suggested that SRP can engage ribosomes even earlier, before the targeting signals on the nascent polypeptide are

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2 The abbreviations used are: TMD, transmembrane domain; ER, endoplasmic reticulum; GET, guided entry of tail-anchored protein; SRP, signal recognition particle; EMC, ER membrane protein complex; TA, tail-anchored membrane protein; TPR, tetratricopeptide repeat; CaM, calmodulin; UBL, ubiquitin-like; PDB, Protein Data Bank.
translated (7). Via interaction with the SRP receptor, SRP delivers translating ribosomes to the Sec61p translocase at the ER membrane (or the SecYEG translocase at the bacterial plasma membrane), often before an additional 60–100 residues of the nascent protein is synthesized (Fig. 1, left path) (8–12). The strictly co-translational nature of the SRP pathway ensures that the nascent TMDs are effectively shielded by proteinaceous environments in either the SRP or the Sec61p (or SecYEG) complex, thus minimizing exposure to the aqueous cytosolic environment during their biogenesis.

Much less is known about the targeting of membrane proteins harboring internal TMDs (Fig. 1, middle path). A genetic screen identified three genetically linked SND (for SRP-independent targeting) proteins, Snd1 in the cytosol, and Snd2 and Snd3 at the ER membrane, whose loss led to mislocalization of this class of proteins (13). More recently, the human orthologue of yeast Snd2 has been described (14). Nevertheless, localized ribosome-profiling data suggested that SRP is responsible for the cotranslational ER localization of most membrane proteins containing internal TMDs (Fig. 1, dashed arrow a (11)). In addition, the SND genes are synthetically lethal with the GET genes (13). These observations suggest that the SND components provide a backup system for the SRP and GET pathways to deliver membrane proteins with relatively downstream TMDs. Analogous diversity is observed with translocases at the ER membrane. The insertion of some SRP-dependent membrane proteins and less hydrophobic TAs are dependent on the ER membrane protein complex (EMC) (Fig. 1) (15–17). In addition, Snd2/Snd3 genetically and physically interacts with Sec72p (13), a component of the post-translational Sec62/63/71/72 translocase conserved across eukaryotic organisms (18, 19). The diversity and redundancy of targeting and translocation machineries are thought to provide a robust network that accommodates the targeting needs of diverse membrane proteins with different TMD location, topology, and charge distribution.

At the other extreme is the class of tail-anchored membrane proteins (TAs), whose TMD is near the C terminus (Fig. 1, right path). TAs comprise up to 5% of the eukaryotic membrane proteome and mediate diverse cellular processes, including protein translocation across organellar membranes, vesicle fusion, apoptosis, and protein quality control (2, 20–22). As the C-terminal TMD is obscured by the ribosome during translation, it was predicted early on that TAs undergo obligatorily post-translational mechanisms of targeting (22). The past decade has witnessed the discovery of several pathways that mediate the targeted delivery and insertion of TAs, including the GET-, SND-, and EMC-dependent pathways (Fig. 1) (2, 6, 13, 16, 20, 21, 23). The GET pathway, which targets relatively hydrophobic TAs to the ER, is especially well-studied. This pathway is also remarkably conserved among eukaryotic cells; all of the components in the yeast GET pathway have orthologues or functional homologs in mammalian cells. The readers are referred to Refs. 2, 6, 20, and 21 for comprehensive reviews of the GET pathway and the targeting of tail-anchored proteins in general. Here, I will focus on the works that uncovered and characterized a multicomponent chaperone system required for the biogenesis of this essential class of membrane proteins.

**A chaperone cascade guides TAs to the ER**

Components of the GET pathway were initially identified through biochemical reconstitutions and genetic interaction
analyses of the secretory pathway in yeast. Work in rabbit reticulocyte lysate identified a 40-kDa ATPase, TRC40, which cross-links efficiently to the C-terminal TMD of model TAs and allows insertion of the bound TA into ER microsomes (24, 25). The yeast homologue of TRC40, Get3, was epistatically linked to two ER-localized membrane proteins, Get1 and Get2 (26), which were subsequently shown to act as both a receptor complex for TA-loaded Get3 and a translocase that mediates TA insertion into the ER membrane (Fig. 2, step 7) (27, 28). As the central targeting factor in the GET pathway, the structure, dynamics, and activity of Get3 have been extensively studied, providing a high-resolution mechanistic model for how this targeting factor couples its ATPase cycle to the ER targeting of TAs (see “Get3: An ATP-driven protean clamp”).

Nevertheless, it soon became clear that TA capture by Get3 (or TRC40) is a facilitated process in the crowded cytosolic environment. Nascent TA released from the ribosome was poorly captured by partially purified TRC40 (29). Using biochemical reconstitutions, Wang et al. showed that the products of two additional genes epistatically linked to Get3, Get4 and Get5, form a scaffold complex that bridges between Get3 and an upstream cochaperone, Sgt2, and facilitates TA transfer from Sgt2 to Get3 (Fig. 2, steps 5–6) (30). In mammalian cytosol, the C-terminal part of the BAG6 complex (composed of BAG6, TRC35, and UBL4A) was shown to be structurally and functionally homologous to Get4/5 and facilitates TA loading onto TRC40 from SGTA, the mammalian Sgt2 homologue (29, 31, 32). Thus, the substrate loading mechanism via the Sgt2-to-Get3 transfer is conserved among eukaryotic cells.

Despite these advances, how newly synthesized TAs are captured by Sgt2 remained a longstanding puzzle. Purified Sgt2 is ineffective in capturing TAs in the soluble form, and attempts to directly load TA onto Sgt2 led to extensively aggregated complexes (23). For many years, generation of soluble, functional Sgt2- or SGTA-containing complexes has relied on cell lysates that contain endogenous chaperone (30, 33) or superphysiological Sgt2/SGTA concentrations (32). Importantly, Sgt2 contains a conserved tetratricopeptide repeat (TPR) domain that associates with multiple heat shock proteins, including Hsp70, Hsp90, and Hsp104 (30, 34, 35). This observation led to the hypothesis that heat shock proteins are further required to facilitate TA loading on Sgt2 (35). Experimental evidence for this model emerged recently through the work of Cho et al. (23), who demonstrated that the major cytosolic Hsp70 in yeast, Ssa1, is highly effective in capturing newly synthesized TAs and efficiently transfers the bound TAs to Sgt2, in a manner dependent on its interaction with the Sgt2 TPR motif. In vivo, transient inactivation of Ssa1 severely disrupted TA insertion into the ER, analogous to observations with GET gene deletions (23). Together, Hsp70, Sgt2, Get4/5, and Get3 form the mini-
mal components that allow reconstitution of the molecular events required to generate a soluble, translocation-competent targeting complex in the cytosol (23).

Collectively, these works demonstrate that even a compositionally simple integral membrane protein, such as the TA, is sequentially funneled through a multicomponent Hsp70-co-chaperone cascade (Fig. 2). Newly synthesized TAs released from the ribosome are captured by Ssa1, which effectively shields the TA-TMD from aggregation in the aqueous cytosol (steps 1–2). Ssa1 assembles the first transfer complex via interaction of its C terminus with the TPR domain of Sgt2, in which TA is rapidly transferred (steps 3–4). A second client transfer complex is assembled via the Get4/5 scaffold complex, which bridges between Sgt2 and Get3 to facilitate TA transfer onto Get3 (step 5). The TA is then delivered to the ER membrane via the interaction of Get3 with the Get1/2 receptors (steps 6–7). Although the complexity of the GET pathway is counterintuitive, many observations in this pathway suggest potential chemical and biological rationales for the evolution of this elaborate chaperone cascade, and their further investigation could provide valuable insights into the roles and organization principles of chaperone networks in general. Below, I highlight and discuss the implications of some of these observations, with the hope to stimulate additional studies into this and conceptually analogous multicomponent chaperone systems.

**Improved client conformational quality via stepwise loading**

What drives the directional substrate transfers in the GET pathway? Quantitative measurements suggested that both the Ssa1-to-Sgt2 and Sgt2-to-Get3 TA transfers are energetically downhill, with the transfer equilibrium ~100- and ~20-fold in favor of the downstream chaperone in the respective transfer complexes (23, 33). This implies that the downstream chaperones bind TAs much more strongly than their respective upstream chaperones, and TA transfer in the reverse direction is unfavorable under physiological conditions. Measurements of the kinetic stabilities of Sgt2-TA and Get3-TA complexes supported this model, showing that their half-times for spontaneous dissociation are ~40 min (23) and ~4 h (33, 36), respectively. Thus, successive substrate transfers in the Hsp70-Sgt2-Get3 triad are thermodynamically driven, with TAs engaging in increasingly stable interactions with chaperones as they progress through the pathway.

If the downstream chaperones bind TAs more tightly, why is participation of Hsp70 necessary? An intriguing observation is that stepwise substrate loading via Ssa1 significantly enhances the conformational quality of TA substrates. Direct loading of TAs on Sgt2 is inefficient and resulted in largely aggregated, inactive complexes. In contrast, Sgt2-TA complexes generated via transfer from Ssa1 are not only soluble, but also functionally competent in undergoing subsequent steps in the GET pathway (23). Although the client interactions of Ssa1 and Sgt2 remain to be studied at higher resolution, kinetic determinants are likely responsible for these observations. The aggregation of single-pass membrane proteins in aqueous environments tends to be rapid (τ < 10 s (23)) and, without external energy input, irreversible. Although Sgt2 and Get3 bind TAs with high kinetic stability, their substrate binding kinetics at physiological concentrations (0.5–1 μM) (37, 38) are probably too slow to compete with TA aggregation. In contrast, Hsp70 binds client proteins rapidly in the ATP state (~10^9 M^-1 s^-1 (39, 40)) and is far more abundant in the cytosol (~15 μM) (37, 38) compared with Sgt2 and Get3. These factors enable cytosolic Hsp70s to more effectively compete with off-pathway misfolding and aggregation processes, allowing nascent TAs to be captured in a soluble, functionally competent conformation. The conformational quality of TAs appears to be effectively preserved during both the Ssa1-to-Sgt2 and Sgt2-to-Get3 handovers, likely through concerted substrate transfer mechanisms (see “Client privilege in the chaperone cascade”). Thus, the sequential substrate loading and transfers in the GET pathway are governed by a combination of thermodynamic forces and kinetic constraints, which together ensure that these hydrophobic proteins are maintained in a soluble, translocation-competent state en route to the ER membrane.

**Client privilege in the chaperone cascade**

Client handover from Hsp70 to downstream chaperones, such as GroEL/ES and Hsp90, is integral for the folding of numerous proteins. Despite the importance of these transfer events, their detailed molecular mechanisms are poorly understood. For example, current models largely assume a passive mechanism in which recalcitrant substrates released from Hsp70 simply diffuse to the GroEL chaperonin to complete their folding (41, 42), and bacterial outer membrane proteins are assumed to associate with and dissociate from multiple periplasmic chaperones before insertion into the membrane (43). Intriguingly, studies in the GET pathway provided convincing evidence for a strongly facilitated, highly privileged client handover mechanism during the Sgt2-to-Get3 (or SGTa-to-TRC40) TA transfer. First, the transfer is kinetically facile, with a halftime of 10–20 s (32, 33). This is >10^2-fold faster than spontaneous TA dissociation from Sgt2 (23), suggesting that the transfer occurs via a more active mechanism than simple TA release and diffusion from Sgt2 to Get3. Further, the transfer is impervious to the presence of off-pathway chaperones that act as a TA trap, such as calmodulin (CaM), whereas the isolated SGTa-TA or Sgt2-TA complex quickly loses the bound TA to CaM (32, 36). These observations strongly suggest that TAs are also physically shielded from alternative chaperones in the cytosol during their transfer from Sgt2 to Get3.

A recent study further highlights that conserved molecular mechanisms have evolved to ensure client privilege during the Sgt2-to-Get3 transfer. A conserved helix 8 (termed a8) lining the substrate-binding groove of Get3, which was unresolved in most crystal structures, was found to specifically promote rapid and privileged TA transfer from Sgt2 to Get3 (36). Mutations of a8 slowed TA transfer from Sgt2 to Get3 ~100-fold and largely abolished the role of the Get4/5 complex. Moreover, Get3 lost its privilege to capture TAs from Sgt2 upon mutation of a8, and the TA substrate was instead lost to external chaperones, such as CaM (36). These defects *in vitro* are corroborated by the enhanced stress sensitivity and TA insertion defects of yeast cells harboring Get3(a8) mutations (36, 44). Coupled with the observation that a8 can cross-link to TAs (44), it was proposed...
that the flexible a8 motif mediates the earliest contacts of Get3 with the TA during its transfer from Sgt2, helping to guide the TA into the substrate binding groove of Get3 while also shielding the TA from off-pathway chaperones during this process (Fig. 3) (36). Importantly, privileged client transfer provides an effective mechanism to not only protect nascent membrane proteins from re-exposure to the aqueous cytosolic environment, but also ensure that substrates are retained within a dedicated biogenesis pathway en route to the target membrane.

The molecular mechanisms that underlie the active and privileged substrate transfers in the GET pathway remain to be determined. On one hand, many individual domains and interactions in the Sgt2-Get4/5-Get3 transfer complex have been extensively studied. Sgt2 (and SGTA) is characterized by a sub-class of TPR domains frequently found in HSC chaperones, including Hsp-organizing protein (HOP in human and Sti1 in yeast) and C terminus of HSC-interacting protein (CHIP) (35, 45–48). Five conserved residues in this TPR domain form a dicarboxylate clamp that recognizes a C-terminal EEVD motif in Hsp70, Hsp90, and Hsp100 (Fig. 3, left inset), linking Sgt2 to multiple protein folding pathways. The N terminus of Sgt2 mediates its homodimerization and forms an interaction platform for the ubiquitin-like (UBL) domain of Get5 (Fig. 3, bottom inset), linking Sgt2 to downstream components of the GET pathway (49–51). At the other end of this transfer complex, Get4 binds with nanomolar affinity to ATP-bound Get3 and bridges the Get3 dimer interface (Fig. 3, right inset) (52, 53). As detailed below (see “Get3: An ATP-driven protean clamp”), the interactions of Get4/5 not only bring Sgt2 and Get3 into close proximity but also optimize the conformation and nucleotide state of Get3 for TA capture. On the other hand, due to the multiple flexible elements in Sgt2 and Get4/5, the organization and architecture of this transfer complex is largely unknown. In addition, the C-terminal domain of Sgt2, rich in glutamine and methionine, forms the binding site for hydrophobic TMDs on TA substrates (Fig. 3, SBD), but the molecular basis of substrate recognition by Sgt2 remains unclear (30).

Whether the upstream Hsp70-to-Sgt2 TA transfer is also privileged and the molecular mechanisms that give rise to privileged client transfer remain outstanding questions. Finally, the client interaction of Hsp70 is extensively regulated by its own ATPase cycles and by chaperones, such as Hsp40, that tune the conformation and nucleotide state of Hsp70; this further raises questions of whether and how additional Hsp70 chaperones are involved in the targeting pathway and the client transfer process. Deciphering the conformation and dynamics of substrate-bound Hsp70 and Sgt2, and how these biophysical properties impact their substrate recognition, are likely key to understanding the kinetic acceleration and privilege of the TA during its transfers in the GET pathway.

Client selection and triage

Whereas the roles of Hsp70 and Get3 in the GET pathway (client capture and targeting to the ER, respectively) are easier to understand, the precise roles of Sgt2 (and SGTA) are less clear. An interesting hypothesis is that this cochaperone provides a mechanism to reject suboptimal substrates from the GET pathway. Co-immunoprecipitation experiments by Wang et al. (30) first showed that Sgt2 can distinguish between TAs destined to the ER versus mitochondria. By systematically varying the TMD in model TAs, biochemical analyses showed that Sgt2 preferentially binds TMDs that have higher hydrophobicity and helical content, features that distinguish GET substrates from mitochondrial TAs (33). Another study, which examined a large set of TAs, further showed that Sgt2 does not efficiently capture TAs with low-hydrophobicity TMDs, which can be inserted into the ER via the alternative EMC pathway (16). Although the preferences of Sgt2 for more hydrophobic TAs are paralleled by Get3 (33), the high kinetic stability of the Get3-TA complex renders it less effective at rejecting suboptimal TAs. The lifetime of Get3 bound to a model GET substrate is 1–4 h, whereas TA insertion into the ER occurs within 5–10 min (33, 36, 54). Thus, most TAs that have been loaded on Get3 are committed to insertion into the ER, and suboptimal TAs
that bound Get3 less tightly do not efficiently dissociate before the insertion. The upstream chaperone in the pathway, Ssa1, is known to promiscuously associate with diverse nascent proteins (55). Sgt2 was therefore proposed to provide a key selection filter that rejects TAs and other membrane proteins destined to alternative organelles or targeting pathways (Fig. 2, dashed arrows in b–e). In the mammalian system, the BAG6 complex that replaces Get4/5 contains an additional UBL domain that recruits the ubiquitin ligase RNF126 and can mediate polyubiquitylation of substrates loaded on SGTA (32, 56), potentially generating an additional branch that triages mislocalized membrane proteins to quality control pathways.

These observations suggest a modular organization of the GET pathway, in which each chaperone/cochaperone fulfills a distinct function that together enable the efficient, selective, and unidirectional targeting of nascent TAs. As nascent proteins are funneled through this cascade, they engage more specialized chaperones with increasingly high affinity and become more committed to insertion into the ER. Analogous functional specialization of downstream cochaperones that collaborate with Hsp70 are well-documented. For example, TPR-containing co-chaperones, Sti1/HOP, mediate the handover of kinase substrates from Hsp70 to Hsp90, enabling Hsp90 to complete the folding of numerous members of the kinase superfamily (57–59). Upon encounter with protein aggregates, Hsp70 could recruit and collaborate with the Hsp100 family of chaperones to refold the aggregated proteins (60–63). More broadly, client handover from Hsp70 to downstream chaperones or cochaperones could provide a versatile triaging mechanism via which distinct classes of client proteins are sorted to their dedicated biogenesis pathways. This hypothesis and the detailed molecular mechanisms of client triage in these chaperone/cochaperone systems remain to be studied.

**Get3: An ATP-driven protean clamp**

At the end of the chaperone cascade, Get3 receives the TA substrates from Sgt2 and delivers them to the ER membrane. As the core targeting factor in the GET pathway that binds TA substrates with extraordinarily high stability, Get3 provides a valuable opportunity to elucidate the client interaction of a membrane protein chaperone at high resolution. As a member of the SIMIBI (after signal recognition particle (SRP), MinD, and BioD) class of nucleotide hydrolases, studies of Get3 also provided insights into the regulatory mechanism of an emerging family of dimerization-activated GTPase and ATPases whose mode of action differs significantly from that of the classic signaling GTases (64–67).

Earlier work has provided beautiful structural illustrations for how Get3 undergoes ATP– and interaction partner–induced conformational rearrangements that can be coupled to substrate binding and release (Fig. 4A, TA-loading phase on the left; see Refs. 2, 20, and 21 for more comprehensive reviews on Get3 structure and function). Get3 is an obligate homodimer in which the ATPase domains directly bridge the dimer interface and are structurally and functionally coupled to a helical domain (Fig. 4B). Early crystallographic work showed that non-hydrolyzable ATP analogues induce adjustments at the dimer interface, which are amplified into larger movements of the helical domains that bring them close to one another (Fig. 4, A (“Closed” Get3) and B (left)) (44, 68–70). Importantly, “closing” brings together conserved hydrophobic residues in the helical domains of Get3 to form a contiguous hydrophobic groove, which provides the docking site for the TA-TMD (71). Get4/5 selectively binds to and stabilizes ATP-bound closed Get3 and further inhibits its ATPase activity (Fig. 4A, Occluded) (53, 72, 73). Presumably, these ATP- and Get4/5-induced rearrangements optimize Get3 for capture of the TA substrate from Sgt2 (Fig. 4A, TA Loading). At the other extreme, the cytosolic domain of Get1 (Get1–CD) form a coiled-coil that inserts like a wedge into the Get3 dimer interface, inducing a wide open conformation of Get3 (Fig. 4, A (Open) and B (right)) (74–76). Get1–CD not only disrupts the TA binding groove of Get3, but also induces both the switch I and switch II loops at the Get3 ATPase site into a conformation incompatible with ATP binding (73–77). These Get1–induced rearrangements are believed to be responsible for triggering the release of TA from Get3 at the ER for membrane insertion.

A dilemma posed by this early model is that an exclusively closed Get3–TA complex would preclude downstream events in the pathway that require Get3 to dissociate from Get4/5. These include the interaction of Get3 with the Get1/2 receptor, whose binding sites on Get3 heavily overlap with that of Get4/5 (53), and ATP hydrolysis by Get3, which is inhibited by Get4/5 (72). More generally, chaperones that engage and deliver membrane proteins not only need to effectively capture substrates, but also need to promptly release the bound substrates at the target membrane. The mechanisms that enable membrane protein chaperones to transition from the substrate-loading mode to the substrate-releasing mode are not well-understood.

The resolution to this dilemma was provided by a more recent single-molecule spectroscopy study, which uncovered unusual substrate-induced dynamic motions in this ATPase and elucidated how these dynamics drive the targeting phase of the GET pathway (Fig. 4A, right). In this work, the open–closed conformational rearrangements of Get3 were directly monitored using a pair of FRET dyes incorporated at its helical domains (Fig. 4B, green and red stars) (54). Contrary to the accepted models, this study found that the TA substrate initiates sub-millisecond time-scale opening motions in Get3 that drive the targeting phase of the pathway (Fig. 4, A (Targeting) and B). Biochemical analyses demonstrated that these substrate-induced dynamic motions led to adjustments at the Get3–Get4 interface that enable more facile dissociation of Get3 from the Get4/5 complex (Fig. 4A, step 5). Once released from Get4/5, the TA substrate activates ATP hydrolysis on Get3 (Fig. 4A, step 6). These biochemical changes are coupled with increasing dynamics of the Get3–TA complex to more extensively sample the open conformation (Fig. 4C) (54). This renders dissociation from Get4/5 irreversible and primes Get3–TA for interaction with and remodeling by the Get1/2 receptors (Fig. 4A, step 7), driving the relocalization of the targeting complex from the cytosol to the ER membrane.

Whereas earlier models of Get3–TA interaction invoked a “lock–and-key” type mechanism in which the TA substrates fit into a preorganized hydrophobic groove on Get3, observations from the single-molecule study suggest a distinct model in
which Get3 forms a rapidly fluctuating “protean clamp” that stably traps substrates. Analogous conformational dynamics of chaperone-client interactions have been observed with multiple ATP-independent chaperones that mediate outer membrane protein biogenesis in the bacterial periplasm (78–80). In addition, although earlier work based on peptide substrates associated a lid-closed conformation of Hsp70 with the high-affinity client-binding state, more recent NMR, EPR, and single-molecule experiments revealed remarkable conformational heterogeneity and dynamics in Hsp70 when it engages full-length protein substrates (81–84). It is conceivable that rapidly fluctuating chaperones and dynamic chaperone-client interactions operate in many systems to retain substrates with high affinity, while also providing functional switches to propel the progression of vectorial pathways.

**Additional Hsp70-cochaperone pairs in membrane protein targeting**

Cytosolic Hsp70s participate in almost every stage of the protein life cycle, from de novo folding and protein aggregate remodeling to protein quality control (42). Recent works further highlighted essential roles of Hsp70s in an increasing number of membrane protein targeting pathways. The participation of Hsp70 in protein transport was initially recognized in studies of secretory and mitochondrial precursor proteins (85–89). However, the extent of Hsp70 participation in protein targeting had been unclear in the earlier work: among nine preprotein substrates examined, the targeting of only two ER- and one mitochondria-destined proteins were affected by Ssa1 inactivation (86). Investigations of the GET pathway added an essential class of integral membrane proteins to the list of clients whose proper cellular localization is directly mediated by Hsp70.

Additional recent studies showed that cytosolic Hsp70 and its associated Hsp40s, Ydj1 and Sis1, interact with and are required for the efficient targeting of β-barrel membrane proteins (90) and a subset of TAs (3) to mitochondria and peroxisomes. Two less abundant Hsp40s, Xdj1 and Djp1, were also found to preferentially bind subsets of mitochondrial membrane proteins, such as Mim1 and Tom22, and promote their biogenesis (91, 92). In the case of Xdj1, the J-domain that binds and regulates Hsp70 was required for substrate import, indicating that it cooperates with Hsp70 to carry out this process. Collectively, these recent observations suggest that cytosolic Hsp70s and/or...
their associated Hsp40s provide a hub that rapidly captures nascent membrane and organellar proteins and facilitate their targeted delivery to diverse intracellular membranes.

Once Hsp70s and/or their associated Hsp40s captures the preprotein substrates, how the substrates are guided to the correct target membranes remains to be elucidated. In the example of the GET pathway, the interaction of the Hsp70 EEVD motif with the TPR domain of Sgt2 provides the mechanism to direct hydrophobic TAs into this pathway. Notably, Hsp70s also bind to the TPR motifs on the mitochondria import receptor Tom70, and this interaction is required for the import of a subset of mitochondrial precursor and β-barrel membrane proteins (88, 90). Additional TPR-containing receptors have been found on the membrane of other organelles, including Pex5 (peroxisomal biogenesis factor 5) involved in the biogenesis of peroxisomal matrix proteins (93, 94), and Sec71/72 that associates with the Sec62/63 translocase to assist the post-translational translocation of precursor proteins across the ER (95). Another class of mechanisms involves the Hsp40s, which not only bind client proteins in cooperation with Hsp70 but also interact with receptors on specific organelles. For example, the major cytosolic Hsp40s, Ydj1 and Sis1, preferentially bind to the mitochondrial import receptor Tom20, providing a redundant import route that works in parallel with the Tom70-mediated pathway. Another Hsp40 involved in Mim1 biogenesis, Xdj1, is a specific interaction partner of Tom22, the central receptor of the translocase of the mitochondrial outer membrane (92). These observations suggest that the functional coupling of Hsp70 with co-chaperones that are either organelle-specific or mediate organelle-specific interactions could provide a general mechanism to direct the localization of nascent membrane proteins to distinct cellular organelles. How diverse nascent membrane proteins are distinguished by the different Hsp70-co-chaperone pairs and thus engage the correct targeting pathway remains an outstanding question that lies at the heart of understanding the fidelity of protein localization.

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