Crystal structure of ATP-bound Get3–Get4–Get5 complex reveals regulation of Get3 by Get4

Harry B Gristick, Meera Rao, Justin W Chartron, Michael E Rome, Shu-ou Shan & William M Clemons Jr

Correct localization of membrane proteins is essential to all cells. Chaperone cascades coordinate the capture and handover of substrate proteins from the ribosomes to the target membranes, yet the mechanistic and structural details of these processes remain unclear. Here we investigate the conserved GET pathway, in which the Get4–Get5 complex mediates the handover of tail-anchor (TA) substrates from the cochaperone Sgt2 to the Get3 ATPase, the central targeting factor. We present a crystal structure of a yeast Get3–Get4–Get5 complex in an ATP-bound state and show how Get4 primes Get3 by promoting the optimal configuration for substrate capture. Structure-guided biochemical analyses demonstrate that Get4-mediated regulation of ATP hydrolysis by Get3 is essential to efficient TA-protein targeting. Analogous regulation of other chaperones or targeting factors could provide a general mechanism for ensuring effective substrate capture during protein biogenesis.

In eukaryotes, the proper targeting of membrane proteins is a major challenge for the cell to overcome. Integral membrane proteins contain hydrophobic transmembrane domains (TMDs), which must be protected from the aqueous cytosolic environment before their integration into the appropriate membrane. For the majority of membrane proteins targeted to the endoplasmic reticulum (ER), this is accomplished by the signal recognition particle (SRP), which typically binds the initial TMD, or signal anchor, as it emerges from the ribosome and targets it to the ER for cotranslational insertion. Exceptions are the ubiquitous TA proteins, defined topologically by a single transmembrane domain near the C terminus, which are unable to access the SRP and must be targeted to the ER post-translationally. For the majority of membrane proteins, this is accomplished by the signal recognition particle (SRP), which typically binds the initial TMD, or signal anchor, as it emerges from the ribosome and targets it to the ER for cotranslational insertion. Exceptions are the ubiquitous TA proteins, defined topologically by a single transmembrane domain near the C terminus, which are unable to access the SRP and must be targeted to the ER post-translationally.

Found in most cellular membranes, TA proteins are targeted to either the ER or the mitochondria. For the latter, a dedicated pathway has not been identified. TA proteins destined for other organelles are initially targeted to the ER and then are subsequently trafficked to the appropriate membrane. Examples include many essential apoptotic or signal transduction proteins such as SNAREs (involved in vesicle fusion), Bcl-2 (involved in apoptosis), and Sec61α (part of the protein translocation machinery). A need for specific targeting of ER-destined TA proteins was first conceptualized nearly two decades ago, and the cellular factors responsible have now been identified. The central targeting factor was identified biochemically in a mammalian system as the cytosolic ATPase TRC40, demonstrated to bind the TA and deliver it specifically to the ER. Previous genetic experiments involving the yeast multidomain Hsp70 or Hsp90 cochaperone, Sgt2 and Get3 (refs. 11,24,26). Structural studies of Get4 and the homolog, Get3, could then be linked to TA targeting, thus providing a route for studying this process. These were followed by a series of results that characterized the new pathway, termed guided entry of TA proteins (GET). In yeast, this pathway includes six proteins, Get1–Get5 and Sgt2, all with homologs in higher eukaryotes.

Structural characterization of Get3, the central TA-targeting factor, demonstrates that it undergoes ATP-dependent conformational changes from an apo ‘open’ form to an ATP-bound ‘closed’ form required for capturing the TA substrate. Deletion of Get3 in yeast leads to a buildup of mislocalized cytosolic TA proteins and is embryonic lethal in mice. The Get3–TA complex is recruited to the ER by the membrane proteins Get1 and Get2. These stimulate release of the TA protein and subsequent insertion into the ER membrane, although the specifics of this mechanism are as yet unknown. Upstream of Get3 is the multidomain Hsp70 or Hsp90 cochaperone Sgt2 (refs. 11,24,25). Sgt2 specifically binds the TA in the first committed step in TA targeting, and this is followed by handover to Get3 (ref. 24).

Efficient delivery of a TA substrate to Get3 requires the heterotetrmeric Get4–Get5 (Get4–5) complex, which provides the link between Sgt2 and Get3 (refs. 11,24,26). Structural studies of Get4 and the N-terminal domain of Get5, also called Mdy2, revealed that Get4 is an α-helical repeat protein, with the N terminus of Get5 wrapping around its C terminus. Biochemical and genetic evidence implicated the N-terminal face of Get4 in Get3 binding at an interface that shared commonalities with the binding sites for Get1 and Get2 (refs. 27,28). In small-angle X-ray scattering reconstructions, the full-length Get4–5 complex forms an extended structure in which Get4 flanks the Get5 ubiquitin-like domain (Ubl) and central Get5 C-terminal homodimerization domain. Initial results suggested that binding of Get4 to Get3 required nucleotide; however, a subsequent publication has brought this into question. More recent work has expanded on the role of Get4–5 in TA targeting beyond acting as a simple bridge. In addition to preferentially recognizing a nucleotide-bound Get3, Get4 inhibits Get3 ATP hydrolysis; thus, Get4 helps to...
stabilize Get3 in a conformation competent for TA binding. Because a major outstanding question is how Get4 regulates Get3 activity, we set out to understand the structural basis of Get4 function.

This report describes the 5.4-Å crystal structure of an ATP-bound Get3–Get4–5 complex from *Saccharomyces cerevisiae*, a combined ~160-kDa heterohexameric structure. We found two functionally distinct binding interfaces for anchoring and ATPase regulation between Get3 and Get4 and confirmed them biochemically and genetically. Mutations at these interfaces demonstrated that Get4–5-mediated regulation of ATP hydrolysis by Get3 was critical for efficient TA targeting. Finally, crystallographic tetramers of Get3 are compatible with two Get3 dimers bridged by a single Get4–5 heterotrimer. In total, this work illustrates how Get4–5 regulates Get3 by priming it for TA loading, a critical step in this important pathway.

RESULTS

Get4–5 binds the ATP-bound state of Get3

It was first important to establish the requirements for forming a stable Get3–Get4–5 complex. Unless noted, Get3 is either wild type or contains an ATPase-inactivating mutation (D57V, referred to as Get3D) that prevents ATP hydrolysis while still allowing ATP binding. Get4–5 is either the wild-type heterotrimer (Get4–5) or a truncation of Get4 (11–290) and the 54-residue N-terminal domain of Get5 (Get5–Get4–5N), similar to that used in a crystal structure of the heterodimer27. As observed previously30, in low salt (10 mM NaCl) a 1:1 complex of Get3D bound to Get4–5N could be generated that was stable by size-exclusion chromatography (SEC) (Fig. 1a). Increasing the salt concentration resulted in a loss of complex formation, such that no complex was detectable at 500 mM NaCl, thus suggesting an electrostatic interaction. At near-physiological conditions (175 mM NaCl), a complex of Get3D–Get4–5 was disfavored, thus suggesting that additional factors were required to stabilize the complex in vitro.

On the basis of previous evidence that suggested a role for nucleotide in complex formation27, we tested a variety of assays to confirm nucleotide stabilization of the Get3D–Get4–5N complex. We performed SEC in the presence of nucleotide, at a salt concentration under which the complex was disfavored (250 mM NaCl) (Fig. 1a,b). For ADP, we observed a clear stabilization of the complex (Fig. 1b), whereas ATP resulted in the most stable complex—results in agreement with previous experiments27,31. Additionally, we quantified pull-down experiments in which we used tagged Get4–5N to precipitate Get3D, with binding presented as a ratio of Get3D to Get4. These experiments confirmed the nucleotide dependence as observed with SEC, demonstrating a preference for ATP-bound Get3D (Fig. 1c). Finally, we measured affinity constants ($K_d$) by isothermal titration calorimetry (ITC) at a physiological ionic strength (150 mM potassium acetate) (Fig. 1d and Supplementary Fig. 1). For apo-Get3D, a $K_d$ was not measurable, thus suggesting that the affinity was less than 10 µM. In the presence of ADP, Get4–5N bound to Get3D with micromolar affinity that increased to ~500 nM with ATP. Collectively, all three assays show that Get4–5 preferentially interacts with ATP-bound Get3 under conditions of physiological ionic strength.

Architecture of the Get3–Get4–5 complex

Although it is clear that Get4 recognizes the ATP-bound state of Get3, the structural details of this interaction were missing. To understand these, we crystallized a complex of ATP, Get3D and Get4–5N and determined a structure to 5.4-Å resolution (Table 1). In the structure, there is a 1:1 ratio of Get3D to Get4–5N. Get3 is in a closed conformation, as anticipated on the basis of ATP being bound14, with the Get4 interaction lying across the dimer interface (Fig. 2). An ‘anchoring’ interface (described below) buries ~920 Å$^2$ surface area, whereas a ‘regulatory’ interface contributes ~400 Å$^2$ to this interaction (Supplementary Fig. 2).

Table 1 Data collection and refinement statistics

| Get3D–Get4–5N–ATP | Apo | ADP | ATP |
|--------------------|-----|-----|-----|
| **Observed** | 1.57 ± 0.28 | >10 | 0.54 ± 0.04 |

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| **Cell dimensions (Å)** | 166.3, 134.5, 84.1 |
| **α, β, γ (°)** | 90, 113.4, 90 |
| **Resolution (Å)** | 30.0–5.4 (6.0–5.4) |
| | 0.05 (0.46) |
| | 7.4 (2.0) |
| | 93.8 (95.9) |
| **Completeness (%)** | 93.8 (95.9) |
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| **Refinement** | 30.0–5.4 (6.0–5.4) |
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At the anchoring interface, Get4 α2 makes extensive contacts roughly parallel to the groove formed by Get3 α10 and α11 (Fig. 3a and Supplementary Fig. 3). This results in an interaction between the invariant residues Phe246 and Tyr250 of Get3 α10 and Tyr30 of Get4 α2 (Fig. 3a,b and Supplementary Fig. 2b,c). In addition to these hydrophobic contacts, multiple highly conserved charged residues are located within this interface (Glu253, Gln257, Glu258, Glu304 and Asp308 of Get3; Arg37 and Arg42 of Get4). In contrast to α2, α1 of Get4 is located on the opposite face relative to Get3, and it makes fewer contacts. However, the N terminus of Get4 α1 is tilted toward Get3, thus placing the conserved charged residues Lys15 and Arg19 in proximity to Get3 α10. Previously, we demonstrated\cite{27} that residues on the N-terminal face of Get4 bound Get3 residues at a similar surface to that demonstrated\cite{20,21} for the ER receptors Get1 and Get2. A number of these residues map to the anchoring interface (Fig. 250 and Glu253 of Get3; Arg19, Tyr30, Arg37 and Arg42 of Get4) (Fig. 3a and Supplementary Fig. 2a).

At the regulatory interface, located on the opposing monomer, the C-terminal end of Get4 α4 packs against the loop following Get3 α3 (Fig. 3a,b and Supplementary Fig. 3). This places several highly conserved complementary charged residues within this interface (Lys69, Lys72 and Arg75 of Get3; Asp74 and Glu81 of Get4). Central to this interaction are the invariant residues Lys69 (Get3) and Asp74 (Get4), which are located opposite to each other.

Mutational analysis of the Get3-Get4 interface

As noted above, the extensive Get3-Get4 interface involves varied contacts to both monomers. We performed ITC and affinity capture assays to determine which residues are essential for binding (Fig. 3 and Supplementary Fig. 1). As expected, alanine substitution of the invariant hydrophobic residues (Phe246 and Tyr250 of Get3; Tyr30 of Get4) dramatically reduced the binding affinity. In addition, several of the conserved charged residues within the anchoring interface (Glu253 and Glu304 of Get3; Arg19, Arg37 and Arg42 of Get4) produced similar effects after their substitution. A moderate binding defect was present with alanine substitutions of the remaining residues of Get3 α10 (Gln257 and Glu258) and of the loop following Get3 α11 (Asp308). Substitution of the remaining residues of Get4 (Lys15, Lys23, Tyr29, Glu31, His33, Gln34 and Arg45 of Get4), which are located farther away from the core of the anchoring interface and have lower sequence conservation, had little to no effect on binding.

At the regulatory interface, several highly conserved basic residues in Get3 (Lys69, Lys72 and Arg75) are in proximity to oppositely charged residues in Get4 (Asp74 and Glu81). Nevertheless, substitution of these Get3 residues by alanine or aspartate has little to no effect on Get4 binding (Fig. 3c). Furthermore, substitution of Get4 Asp74 by lysine resulted in a marginal increase in affinity as indicated by spheres colored by phenotype (blue, orange, red and white). The positions of side chains cannot be determined at this resolution and are shown here only for reference. Bottom, overview of Get3-Get4–5N in the same orientation used to show the interface. The area within the box represents the interface shown above. (c) Sequence alignments of regions involved in contacts in the Get3-Get4 interface, colored on the basis of ClustalW output\cite{34}. Sc, S. cerevisiae; Af, Aspergillus fumigatus; Ca, Caenorhabditis elegans; XI, Xenopus laevis; Hs, Homo sapiens. Helices are indicated above the sequence and labeled. Residues tested are indicated by spheres colored by phenotype (blue, none or minimal; orange, moderate; red, strong). (e) Summary of results from ITC and pulldown experiments. Mutants are colored by strength of phenotype as in a and b. ITC data were generated from a single experiment; pulldown experiments were performed in triplicate, with the mean shown. Mutants with asterisks are used in Figure 4a. Reg, regulatory.
Figure 4 Get4–5 regulates Get3 ATPase activity. (a) Get3 ATPase assay in the presence and absence of Get4–5 and mutants. The Get4–5 effect is represented as a ratio of $k_{cat}$ in the absence/presence of Get4–5, with a value of 1 indicating no inhibition by Get4–5. The values are shown as mean ± range from independent trials (n specified in Supplementary Table 1). (b) Comparison of Get3 translocation efficiency between wild type (WT) and K69D. (c) Stereo view of the regulatory interface showing interactions between Get4 (blue) and Get3 (purple). (d) Spot-plate growth assays of pRS316-derived rescue plasmids under control of genetic promoters in the BY4741 GET3::KanMX or GET4::KanMX background. KO represents transformation with empty vector. Plates consisted of synthetic complete medium lacking uracil (SC–Ura) with or without 2 mM CuSO$_4$. Each image was taken from a single plate at either 24 h (30 °C, SC–Ura) or 48 h (40 °C, SC–Ura) with 2 mM CuSO$_4$.  

**Regulation of Get3 nucleotide hydrolysis**

Although the regulatory interface is not involved largely in binding, the high conservation of residues at this interface suggests that they have an alternative role in TA targeting. Recently published work demonstrated that Get4–5 binding results in inhibition of Get3 ATP hydrolysis$^{31}$. To test whether this interface has a role, we determined $k_{cat}$ for several mutants of Get3 in the absence and presence of Get4–5 (Supplementary Table 1). A larger value of the ratio of $k_{cat}$ in the absence to that in the presence of Get4–5 indicates inhibition by Get4–5; for example, wild-type Get4–5 inhibits Get3 by approximately six-fold (Fig. 4a). Consistently with their binding defects, the Get3 mutants E253K and E304K were not inhibited by Get4–5 (Fig. 4a). Notably, Get3 K69D, situated at the regulatory interface, lost the ability to be inhibited by Get4–5 (Fig. 4a), although it bound Get4–5 with similar affinity to that of wild type (Fig. 3c). A Get3 K72D mutant, relative to wild-type, also lost the ability to be inhibited by Get4–5, although the loss was to a smaller extent (Supplementary Table 1). Mutation of the invariant Get4 Asp74, situated opposite Get3 Lys69 (Figs. 3b and 4c and Supplementary Figs. 2b,c and 3), yielded the same phenotype (Figs. 3c and 4a). Importantly, combining both opposing mutants (Get3 K69D and Get4 D74K) restored the ability of Get4–5 to regulate Get3 ATPase activity, thus demonstrating that these two residues directly interact (Fig. 4a). This is again consistent with the high conservation of residues located on either side of this interface (Fig. 3b and Supplementary Fig. 2b,c). These results demonstrate that Get4 has two distinct roles for Get3, recruitment and regulation, which can be biochemically decoupled.

To test whether the regulation of Get3 ATPase activity is important for TA targeting, we used a reconstituted *in vitro* targeting assay$^{31}$ in which a TA substrate, Sbh1, is translated in *Δget3* yeast extracts and targeted to ER microsomes by exogenously added Get3. The efficiency of targeting is then reported by the glycosylation of an engineered opsin tag on Sbh1 upon insertion into microsomes. Mutant Get3 K69D, relative to wild type, exhibits an ~40% loss of Sbh1 insertion, a result that agrees with its loss in Get4–5–induced regulation of ATPase activity (Fig. 4a,b and Supplementary Fig. 4a,b). Importantly, this effect is seen only in the presence of Get4–5 because both wild-type Get3 and Get3 K69D have the same targeting efficiency with translation extracts from a *Δget3 Δmdy2* strain (in which Get4 is also depleted$^{24}$) (Supplementary Fig. 4b,c). This is distinct from Get1–2 binding mutants because the critical E253K mutant (that cannot bind Get1 or Get2$^{20–22}$) completely abolishes insertion in both *Δget3* and *Δget3 Δmdy2* extracts (Supplementary Fig. 4b,c); this demonstrates that the Get3 K69D mutant does not directly affect the membrane-associated steps. The formation of functional Get3–TA complexes probably follows a mechanism similar to that of wild type in these mutants because the data still fit a Hill coefficient of 2, a value previously shown to correlate with Get3 tetramer formation$^{31}$. In addition, the targeting by Get3 K69D cannot be rescued by increasing protein concentration (Fig. 4b), results in agreement with a model in which premature ATP hydrolysis in this mutant reduces the fraction of productive Get3–Get4–5 complexes that can capture and target the TA substrate. Thus, Get4–5–induced delay of ATP hydrolysis from Get3 is integral for ensuring efficient TA-protein targeting.

To examine whether this regulation is important for Get3 function *in vivo*, we tested the ability of Get3 K69D and Get4 D74K to rescue known knockout phenotypes by using a yeast growth assay$^{9,13,27}$. As before, neither the *Δget3* nor the *Δget4* strains showed a phenotype when grown on synthetic complete medium at 30 °C. However, growing these strains at 40 °C in the presence of 2 mM Cu$^{2+}$ produced a strong phenotype that could be rescued by expression of the wild-type protein on a plasmid (Fig. 4d). A Get3 K69D mutant was unable to fully rescue the growth phenotype, thus supporting a role for regulation *in vivo*. A Get4 D74K mutant was also unable to fully rescue, resulting in an even stronger phenotype than that of the *Δget3 K69D* mutant (Fig. 4d). It is important to note that this surface of Get4 has no other known interacting partners, meaning that this phenotype can only be a readout of the regulatory role of Get4. In total, these results provide strong evidence that regulation of Get3 ATPase activity by Get4–5 is critical for a functional GET pathway.

**DISCUSSION**

The structure of the Get3–Get4–5 complex presented here reveals the molecular basis of Get3 recognition by Get4. In particular, the structure provides insight into the role of nucleotide in complex formation, in which Get4 binds to both monomers of Get3 in an orientation compatible with only a closed Get3. The anchoring interface mediates the interaction between Get3 and Get4, whereas the regulatory interface is critical for inhibition of Get3 ATP activity. This regulation of Get3 is necessary for efficient targeting *in vitro*, and loss of regulation leads to growth defects *in vivo*. Although it is difficult to speculate...
at subatomic resolution, it is interesting to note that Get3 Lys69 connects through a short helix to the critical switch I loop that contains the catalytic Asp57. This would make the interaction between Get4 Asp74 and Get3 Lys69 allosteric and would lead to inactivating conformational changes in the catalytic pocket.

There is growing evidence that the soluble Get3–TA complex contains a tetramer of Get3, in which two copies of the dimer form a hydrophobic chamber. A tetramer of Get3 is observed in the Get3–Get4–5N crystal lattice in two orientations, colored as in Figure 2b. The tetramer of an archaeal Get3 (PDB 3UG6 (ref. 18)) oriented similarly to a. A model for the assembly of the Get3–Get4–5–TA-binding complex. Colors correspond to those in Figures 2 and 5.

Figure 5  A working model for Get4–5. (a) The tetramer of Get3 from the Get3D–Get4–5N crystal lattice in two orientations, colored as in Figure 2b. (b) The tetramer of an archaeal Get3 (PDB 3UG6 (ref. 18)) oriented similarly to a. (c) A model for the assembly of the Get3–Get4–5–TA-binding complex. Colors correspond to those in Figures 2 and 5.

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ONLINE METHODS

Protein cloning, expression and purification. The sequences of Get4 and Get6 were cloned as previously described22. To generate the Get4–5N used in this study, this construct was further modified by truncation of the C terminus of Get4 (residues 291–312) and by the addition of a stop codon after residue 54 within Get6. All S. cerevisiae Get4 mutants were generated with the QuickChange mutagenesis method (Stratagene) and verified by DNA sequencing. All Get5–5 were cloned as previously described27. To generate the Get4–5N used in this study, this construct was further modified by truncation of the C terminus

Purified Get3D–Get4–5N complex was concentrated to 20 mM Tris, pH 7.5, 10 mM NaCl and 5 mM β-mercaptoethanol (BME). A second Ni-NTA column was used to remove any remaining histidine-tagged protein; then the sample was then loaded onto a 6-ml Resource Q anion-exchange column (GE Healthcare). The peak containing the Get4–5N complex was collected and concentrated to 15–20 mg/ml. Initial purifications of the Get4–5N complex were verified to be a single monodispersed species over SEC with a Superdex 200 16/60 column (GE Healthcare) equilibrated with 20 mM Tris, pH 7.5, 100 mM NaCl and 5 mM BME. Full-length Get4–5 was used in ATPase assays and translocation experiments was further purified with a Superdex 200 16/60 column (GE Healthcare) equilibrated with 20 mM K-HEPES, pH 7.5, 150 mM KOAc, 10 mM MgOAc, 10% (v/v) glycerol and 5 mM BME.

Fractons containing Get4–5 were pooled and concentrated to ~5 mg/ml.

The S. cerevisiae Get3 coding region was cloned as previously described13. A His6 tag followed by a tobacco etch virus (TEV) protease site was fused to the N terminus, and a stop codon was placed in front of the C-terminal His6 tag. All S. cerevisiae Get3 mutants were generated with the QuickChange method. Get3 mutants used in SEC, ITC, or capture assays were introduced into the Get3D construct, whereas mutants used in ATPase assays or translocation assays were introduced into the wild-type Get3 construct. All Get3 proteins were made in BL21-Gold(DE3), grown in 2× YT medium and induced with 0.5 mM IPTG for 10–12 h at 37°C. Cells were lysed with a microfluidizer (Microfluidics) and purified as a complex by nickel-affinity chromatography (Qiagen). The affinity tag was removed by an overnight TEV protease digest at room temperature while dialyzing against 20 mM Tris, pH 7.5, 30 mM NaCl and 5 mM BME. No solution was found with the open (apo) form of Get3 (PDB 3A37 (ref. 17)). Refinement was performed with REFMAC v6.3 with rigid-body restraints and in CNS v1.2 (ref. 41) with DEN refinement. Manual rebuilding was performed with COOT24. The final model was refined to an R factor of 27.0% (Rfree = 32.8%) with residues in the Ramachandran plot with 92.2% in preferred, 6.0% in allowed, and 1.8% in disallowed and restricted regions42. Full statistics are in Table 1.

Size-exclusion chromatography for complex stability. 250 µL of 25 µM Get3D and 250 µL of 25 µM Get4–5N were combined and dialyzed at room temperature in 20 mM Tris, pH 7.5, 10 mM NaCl, 5 mM BME, 1 mM MgCl2, and, where indicated, 1 mM of either ADP or ATP. The total samples were injected onto a Superdex 200 10/300 column (GE Healthcare) equilibrated in 20 mM Tris, pH 7.5, 10–500 mM NaCl and 5 mM BME.

Capture assay. 500 nMol of His6-tagged Get4–5N was incubated with 10 µL Ni-NTA agarose resin for 1 h at 4°C in 500 µL binding buffer containing 20 mM K-HEPES, pH 7.5, 150 mM KOAc, 10 mM MgOAc, 10% (v/v) glycerol, 25 mM imidazole, and 1 mM ADP or ATP where indicated. After the addition of 1 µM of Get3D the solution was incubated for 1 h at 4°C. After incubation, the reaction was spun for 30 s at 500g. The supernatant was removed, and 500 µL binding buffer was added to the solution and gently mixed through inversion. The wash step was repeated twice, and after the final wash the remaining bound proteins were eluted with 30 µL of 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM BME and 300 mM imidazole. The samples were spun for 30 s at 500g, and the supernatant was removed and added to 6 µL of 6x SDS-PAGE buffer. All samples were run on 15% SDS-PAGE gels and stained with Coomassie blue G-250. Gels were then analyzed by infrared scanning in the 700-nm channel with a LI-COR Odyssey Infrared Imaging System and Odyssey Application Software v3.0.30.

Isothermal titration calorimetry. Get3D–Get4–5N binding experiments were carried out with the MicroCal iTC200 system (GE Healthcare). Binding affinities were measured by filling the sample cell with 30 µM Get3D and titrating 350 µM Get4–5N. The buffer conditions were identical for all samples and contained 20 mM K-HEPES, pH 7.5, 150 mM KOAc, 10 mM MgOAc, 10% (v/v) glycerol and 1 mM ATP. For each experiment, 2 µL of Get4–5N was injected into Get3D for 20 intervals spaced 120 s apart at 25°C. For the first titration, 0.4 µL of Get4–5N was injected. The stirring speed and reference power were 1,000 r.p.m. and 5 µcal/s. Affinity constants were calculated from the raw data with Origin v7.4 software (MicroCal).

ATPase assay. Get3 ATPase rates were measured as previously described31. Briefly, the kcat for 8 µM Get3 was determined in the presence of excess of ATP doped with [γ-32P]ATP and analyzed by autoradiography. Each Get3 ATPase reaction was conducted in the presence or absence of excess (20 µM) full-length Get4–5. For Figure 4a, individual ratios were calculated for each of n independent trials (Supplementary Table 1) performed on separate days, and then a mean and s.d. were calculated across n ratios. Each independent trial was the average of values from two side-by-side reactions. Values used in Supplementary Table 1 are means and s.d. calculated across n experiments.

Translocation assay. The coding sequence for yeast Shb1 was cloned into a transcription plasmid32 under control of an SP6 promoter and modified as previously described31. Shb1 mRNA was transcribed with the SP6 Megascript kit (Ambion). All translation and translocation assays were performed in yeast in previously described13–26 with extracts and microsomes from either a Δget3 (ref. 9) or Δget5 Δget7 (ref. 24) strain. Get3 translocation efficiency was plotted as a function of Get3 concentration and analyzed as previously described31.

Yeast growth assay. Knockout strains BY4741 YDL100w::KanMX (Get3) and BY4741 YOR164c::KanMX (Get4) were purchased from American Type Culture Collection (ATCC) and used as previously described13,27. The Get3 rescue plasmid was constructed by PCR amplification of the open reading frame

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with flanking regions 242 bp upstream and 263 bp downstream, from BY4741 genomic DNA. The Get4 rescue plasmid was constructed by PCR amplification of the open reading frame with flanking regions 233 bp upstream and 86 bp downstream, from BY4741 genomic DNA. Both genes were amplified with SalI and NotI restriction sites and ligated into the pRS316 vector. Mutants were generated by QuikChange site-directed mutagenesis. Yeast strains were transformed with the LiAc/single-stranded carrier DNA/PEG method. Phenotypic rescue was determined by growing each transformant in SC–Ura medium at 30 °C to an OD600 of between 1 and 2, diluting to 3.85 × 10⁶ cells/mL and spotting 4 µL of serial dilutions onto SC–Ura agar plates in the presence or absence of 2 mM CuSO₄. Plates were then incubated at 30 °C or 40 °C for 24–48 h and photographed. The results were consistent through three trials.

Structure analysis and figures. Cartoon representations of protein structures were prepared with PyMOL (http://www.pymol.org/), and surface representations were prepared with UCSF Chimera. Surface figures were made in Chimera. Conservation used values for individual residues on the basis of an alignment from ClustalW. Electrostatic surface potentials were calculated with APBS with default values as implemented in the PDB2PQR webserver.

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