Supporting Information

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SI Materials and Methods

Protein Expression and Purification. All mutant proteins were generated using Quikchange Mutagenesis protocol (Stratagene) and purified identically to the WT protein. HIS_6-tagged Get1-CD and Get2-CD (both in a pET33b expression vector) were induced at log phase for 3 h at 37 °C with 0.8 mM isopropyl β-D-1-thiogalactopyranoside. Overexpressed protein in clarified lysate was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography followed by thrombin digestion to remove the HIS_6 tag. Get2-CD was further purified by gel filtration chromatography on a Superdex200 column (GE Healthcare). Get1-CD was further purified by gel filtration chromatography on a superose 12 column (GE Healthcare). HIS_6-tagged mini-Get1/2 and mini-Get1/2RERR (1) were expressed for 3 h at 37 °C and purified by Ni-NTA affinity chromatography. To obtain stoichiometric complexes, partially purified proteins were further purified with a 125-mL Superdex75 gel filtration column (GE Healthcare). Full-length Get1 and Get2 were expressed identically to those in ref. 2 using the overnight autoinduction system (Novagen) in terrific broth (TB) media (3). For purification, Get1 and Get2 (20–30 g dry weight cell pellet) were resuspended in buffer A (50 mM Hepes, pH 8.0, 500 mM NaCl, 10% glycerol) containing protease inhibitor mixture (Roche) and lysed by French press. Unbroken cells were removed by centrifugation at 12,000 × *g* for 20 min, and the supernatant was ultracentrifuged (Ti45; 200,000 × *g* for 50 min). The resulting membrane pellet was washed in buffer A, resuspended in buffer A with 0.5% lauryldimethylamine-oxide (LDAO) and 20 mM imidazole using a dounce homogenizer, and incubated for 1 h under gentle agitation. Detergent-solubilized membrane was clarified by ultracentrifugation in a Ti45 as above, and the supernatant was subjected to Ni-NTA chromatography in buffer A with 0.1% LDAO and 20 mM imidazole. After extensive washing, HIS_6-tagged Get1 or Get2 was eluted in buffer A with 0.1% LDAO and 200 mM imidazole and immediately flash frozen in liquid nitrogen. All proteins (with the exception of full-length Get1 and Get2) were exchanged into Get3 assay buffer in the gel filtration step.

Fluorescence Labeling. Get4/5 (C177T/S48C) was labeled with thiol-reactive acrylodan. Get1-CD–Q62C and Get2-CD–T34C were labeled with maleimide derivatives of either coumarin (DACM) or fluorescein. Proteins were dialyzed in labeling buffer (50 mM KHEPES, pH 7.0, 300 mM NaCl) and treated with 2 mM TCEP to reduce the disulfide bonds. The labeling reaction was carried out using a 10- to 30-fold excess of dye over protein. The reaction was incubated overnight at 4 °C and stopped by adding 2 mM DTT. Excess dye was removed by gel filtration using Sephadex G-25 (Sigma) (4).

Fluorescence Measurements.

Get4/5 equilibrium measurements. Measurements using acrylodan-labeled Get4/5 were based on an environmental sensitive readout. Samples were excited at 370 nm, and fluorescence emission at 490 nm was monitored. For all titrations, Get4/5FL was held constant (50–200 nM), and Get3 concentration was varied. Incubation time was 10 min, and nucleotide was present at 2 mM wherever applicable. Observed fluorescence values (F_{obsd}) were plotted as a function of Get3 concentration and fit to Eq. **S1**,

$$F_{obsd} = F_0 + F_1 \times \frac{K_d + [Get4/5] + [Get3] - \sqrt{(K_d + [Get3] + [Get4/5])^2 - 4[Get3][Get4/5]}}{2[Get4/5]}, \quad \textbf{[S1]}$$

in which F_{obsd} is the observed fluorescence, F_0 is the initial fluorescence value, F_1 is the maximum fluorescence change at saturating Get3 concentrations, and K_d is the equilibrium dissociation constant of the complex.

Get4/5 association and dissociation kinetics. All rate measurements were performed on a Kintek stopped flow apparatus. For association rate measurements, acrylodan-labeled Get4/5 was held constant at 0.2 μM, Get3 concentration was varied as indicated, and ATP was present at 2 mM. Observed rate constants (k_{obsd}) were plotted as a function of Get3 concentration and fit to Eq. **S2**,

$$k_{obsd} = k_{on}[Get3] + k_{off}, \quad \textbf{[S2]}$$

in which k_{on} is the association rate constant, and k_{off} is the dissociation rate constant.

For dissociation rate measurements, a pulse-chase experiment was used. A complex between acrylodan-labeled Get4/5 (at 0.15 μM) and Get3 (at 0.3 μM) was preformed by incubation in 2 mM ATP for 10 min followed by the addition of unlabeled Get4/5 at 6 μM as the chase to initiate Get3–Get4/5 dissociation. The time course for change in fluorescence (F_{obsd}) was fit to a double exponential function (Eq. **S3**),

$$F_{obsd} = F_e + \Delta F_1 \times e^{-k_{fast}t} + \Delta F_2 \times e^{-k_{slow}t}, \quad \textbf{[S3]}$$

in which F_e is the fluorescence when the reaction reaches equilibrium, ΔF_1 and k_{fast} are the magnitude and rate constant of the fluorescence change in the fast phase, and ΔF_2 and k_{slow} are the magnitude and rate constant of the fluorescence change in the slow phase.

Equilibrium measurements of Get3 binding to Get1-CD and Get2-CD. Measurements using fluorescein-labeled Get1-CD or Get2-CD were based on a fluorescence anisotropy readout (Fig. 3). Samples were excited at 450 nm, and fluorescence emission at 518 nm was monitored. For all titrations, Get1-CD or Get2-CD was held constant at 200 nM, and Get3 or Get3/TA concentration was varied. Incubation time was 5–10 min depending on protein concentration, and nucleotide was present at 2 mM. Observed anisotropy values were plotted as a function of Get3 concentration and fit to Eq. **S1**, with anisotropy values replacing the fluorescence values.

Equilibrium measurements of Get3/TA binding to Get1/2-mini. Measurements are based on the fluorescence change of DACM-labeled Get1 (denoted by *) in Get1*/2-mini. Samples were excited at 380 nm, and fluorescence emission at 470 nm was monitored. For all titrations, Get1*/2-mini was held constant at 200 nM, and Get3/TA concentration was varied. Incubation time was 10 min. Observed fluorescence values (F_{obsd}) were plotted as a function of Get3/TA concentration and fit to Eq. **S1**.

Get1-CD dissociation kinetics from Get3. Measurements used a pulse-chase setup on the stopped flow apparatus (Kintek); 150 nM DACM-labeled Get1-CD was preincubated with 350 nM Get3 for 15 min and chased by addition of either 2 mM ATP or 8.5 μM unlabeled Get1-CD to initiate complex dissociation. Samples were excited at 380 nm, and fluorescence emission at 470 nm was

monitored. The time course for change in fluorescence (F_{obsd}) was fit to Eq. **S3**.

Kinetics of mant-ATP dissociation from Get3. Measurements are based on FRET between mant-ATP and a native tryptophan in Get3 (5) using a pulse-chase setup on a stopped flow apparatus (Kintek). A complex between mant-ATP (at 15 μM) and Get3 (1.5 μM) was preformed by incubation for 20 min followed by addition of either Get1 (at 2.5 μM) or excess ATP (2 mM) to initiate complex dissociation. The time course for change in donor (mant-ATP) fluorescence (F_{obsd}) was fit to Eq. **S3**.

Reconstitution of PLs. PLs containing Get1, Get2, or the Get1/2 complex were prepared as previously described (2) with modifications. The following mixture was assembled in a volume of 300–400 μL: 2–3 μM membrane protein, 87 mg washed and degased biobeads (SM-2; Bio-Rad), 30 μL 20 mg/mL lipids (4:1 phosphatidylcholine:phosphatidylethanolamine), and 300 μL reconstitution buffer (50 mM KHEPES, pH 7.4, 500 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 1 mM DTT, 0.25% deoxy-BigCHAP). The mixture was incubated overnight with gentle agitation at 4 °C. For Get2-PL, additional optimization was needed for efficient protein incorporation. The following adjustments yielded robust reconstitution: 600-μL volume protein/lipid mixture (at the concentrations noted above) in reconstitution buffer was incubated with 40 mg biobeads overnight followed by a second incubation with 87 mg freshly added biobeads for 2 h. After biobead removal, 5 volumes cold water was added to the reaction and then pelleted at 311,000 × *g* for 30 min. The resulting PL-containing pellet was resuspended in 70–200 μL membrane buffer (50 mM KHEPES, pH 7.4, 100 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 1 mM DTT). PL concentration was determined by SDS/PAGE with a known amount of recombinant Get1 or Get2 protein using silver stain (Thermo). Note that Get1 stains much more strongly than Get2 by silver stain.

PL Sedimentation Assay. For Get1-PL binding assays, 20 nM Get1-PL or an equivalent volume of empty PL was mixed with 40 nM Get3 or Get3/TA in Get3 assay buffer and 2 mM ATP (where indicated) in a total volume of 150 μL. For Get2-PL binding assays, 96 nM Get2-PL or an equivalent volume of empty PL was mixed with 500 nM Get3 or Get3/TA in Get3 assay buffer and 2 mM ATP (optional) in a total volume of 150 μL. The reactions were incubated for 10 min at room temperature and then ultracentrifuged at 434,000 × *g* for 30 min. The resulting pellet was resuspended in gel loading dye and analyzed by SDS/PAGE and silver staining. Protein bands were quantified using Image QuantTL (GE Healthcare). All values were subtracted from the control with empty PL (set as zero) and normalized to the strongest binder (set as 100%) in each dataset. Comparing Get3 with Get3/TA binding, the values obtained for Get3/TA were multiplied by one-half to account for the fact that Get3/TA is a tetramer, whereas Get3 is a dimeric.

Get3 Recycling Assay. Experiments using Get1-PL (Fig. 7*A*) were initiated by preincubating the following mixture for 10 min at room temperature: 100 nM Get1-PL or an equivalent volume of empty PL, 100 nM HIS_6-Get3, and ATP (at indicated concentrations) in a total volume of 150 μL in Get3 assay buffer. Get4/5 (at indicated concentrations) was then added for an additional 10 min, and the reaction was pelleted as described for the sedimentation assay. Get3 was detected by Western blotting using an anti-His antibody (Qiagen). Experiments with yRM were initiated by preincubating the following mixture for 10 min at room temperature: 0.16 A_{280}/mL Δ*get3* yRM, 50 nM HIS_6-Get3, and ATP (at indicated concentrations) in a total volume of 150 μL in Get3 assay buffer. Get4/5 (at indicated concentrations) was then added for an additional 10 min, and the reaction was ultracentrifuged (TLA100; 436,000 × *g* for 10 min). Equivalent amounts of the soluble and pellet fractions were analyzed by Western blot against HIS.

Kar2 Secretion Assay. Kar2 secretion assays were carried out as described in ref. 6. For Western blot analysis, Kar2 anti-rabbit antibody was used at 1/3,000 dilution. The protein bands were quantified using Image QuantTL (GE Healthcare).

Insertion Assays. All in vitro translation and translocation assays were carried out as described in ref. 5. The substrate used was full-length yeast Sbh1 containing a C-terminal opsin tag. For the experiments in Fig. S4, [^{35}S]methionine-labeled Sbh1 was translated in yeast extracts. Get3/Sbh1-opsin targeting complexes were purified from the extract using an HIS_6 tag on Get3 and presented to 4 A_{280}/mL yRM or PLs at indicated concentrations for 40 min at 26 °C. Completed reactions were prechilled on ice for 25 min and treated with 2 μL 1 mg/mL proteinase K in a 20 μL volume for 10 min. Digestions were quenched with 5 mM PMSF followed by resuspension in boiling 2× SDS gel loading dye. Reactions were analyzed by SDS/PAGE and autoradiography. For the experiments in Fig. S2, recombinant Get3 was coexpressed with HIS_6-Sbh1-opsin (5). The complex was purified as described (5) and incubated with yRM at indicated concentrations in Get3 assay buffer and 4 mM ATP (if indicated) for 45 min at 26 °C. The reaction was loaded on a 50-μL sucrose cushion (0.5 M in assay buffer) and separated into supernatant and pellet fractions by ultracentrifugation at 434,000 × *g* for 30 min. Sbh1 was detected by anti-HIS Western blot, and the glycosylation product was verified by Endo H treatment following the manufacturer's instructions (P0702; NEB).

1. Wang F, Whynot A, Tung M, Denic V (2011) The mechanism of tail-anchored protein insertion into the ER membrane. *Mol Cell* 43(5):738–750.
2. Mariappan M, et al. (2011) The mechanism of membrane-associated steps in tail-anchored protein insertion. *Nature* 477(7362):61–66.
3. Studier FW (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 41(1):207–234.
4. Zhang X, Kung S, Shan S-O (2008) Demonstration of a multistep mechanism for assembly of the SRP x SRP receptor complex: Implications for the catalytic role of SRP RNA. *J Mol Biol* 381(3):581–593.
5. Rome ME, Rao M, Clemons WM, Shan S-O (2013) Precise timing of ATPase activation drives targeting of tail-anchored proteins. *Proc Natl Acad Sci USA* 110(19):7666–7671.
6. Schuldiner M, et al. (2008) The GET complex mediates insertion of tail-anchored proteins into the ER membrane. *Cell* 134(4):634–645.

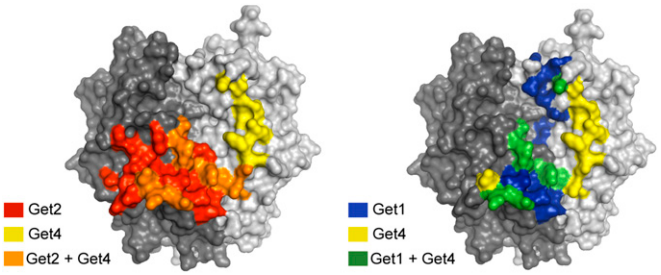
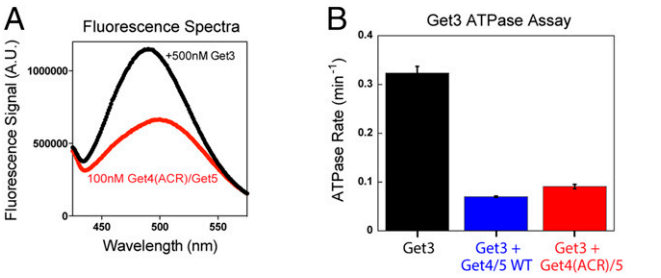
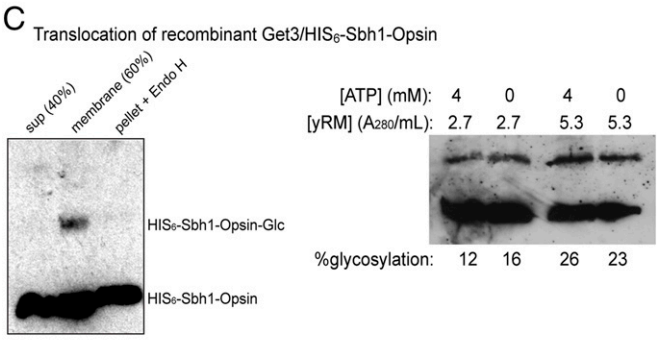


Fig. S1. Overlay of the binding sites of (*Left*) Get4 and Get2 and (*Right*) Get4 and Get1 on the Get3 dimer (1).

1. Gristick H, et al. (2014) Crystal structure of ATP-bound Get3-Get4-Get5 complex reveals regulation of Get3 by Get4. *Nat Struct Mol Biol* 21(5):437–442.





D Comparison of translocation efficiency of Get3/TA complex

	recombinant (this work)	*in-vitro* translated (ref. 12)
a. substrate loaded	33 – 67 nM	33 nM
b. % insertion	16 / 5.3 A_{280} yRM	25 / 10 A_{280} yRM
[TA] inserted* (= a x b)	100-200 nM / A_{280} yRM	83-170 nM / A_{280} yRM

*Estimates are accurate within two-fold. The range associated with the recombinant complex arise from uncertainty in the number of TA substrate bound in each complex. The range associated with the in vitro translated complex arise from potential loss of the linear increase in translocation efficiency at yRM concentrations above 5.3 A_{280} units.

Fig. S2. Interaction of Get3 with Get4/5 (related to Fig. 1). (*A*) Fluorescence emission spectra of 100 nM acrylodan-labeled Get4/5 with (black) and without (red) 500 nM Get3. (*B*) Multiple-turnover ATPase assay with 2.0 μM Get3 alone or in complex with 8.0 μM WT (black) or acrylodan-labeled (red) Get4(C177T, S48C)/Get5. All assays contained 200 μM ATP. (*C*) Bacterially expressed recombinant Get3/HIS_6-Sbh1-opsin complex was presented to yRM for insertion. (*Left*) Verification of translocated (glycosylated) Sbh1-opsin band in the membrane fraction and its sensitivity to endoH treatment. (*Right*) Quantification of the insertion of the recombinant Get3/HIS_6-Sbh1-opsin complex into various amounts of yRMs. The yRM-associated fraction is shown (*Materials and Methods*). (*D*) Comparison of the amount of TA inserted for the Get3/TA complex generated recombinantly (this work) and that generated by in vitro translation (1). A.U., arbitrary unit; Sup, supernatant.

1. Mariappan M, et al. (2011) The mechanism of membrane-associated steps in tail-anchored protein insertion. *Nature* 477(7362):61–66.

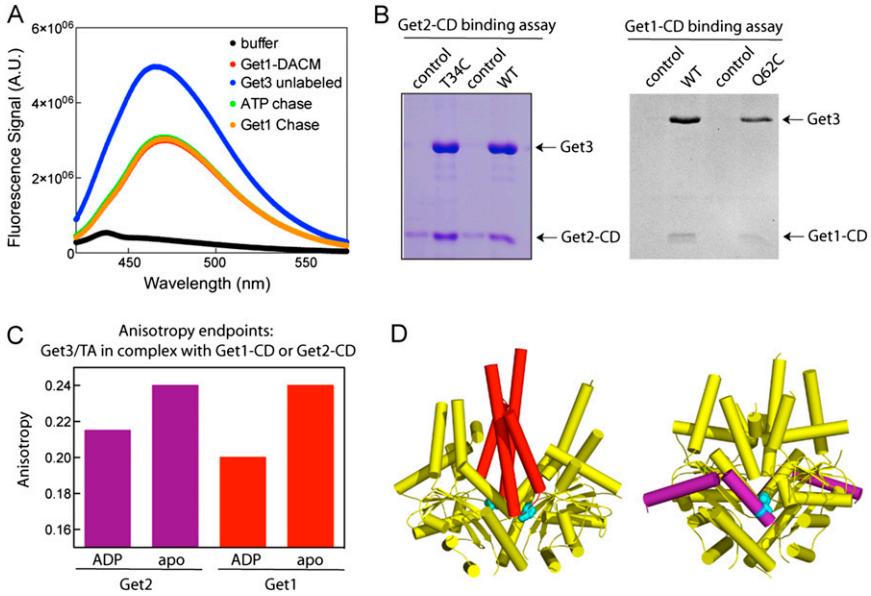


Fig. S3. The interaction of Get3 with Get1-CD and Get2-CD (related to Fig. 3). (*A*) Fluorescence emission spectra of 150 nM DACM-labeled Get1-CD alone (red) or in the presence of the following: 400 nM Get3 (blue), 400 nM Get3 and 2.3 mM ATP (green), or 400 nM Get3, 2.3 mM ATP, and 17 μM unlabeled Get1 (orange). (*B*) Pull-down assay of HIS_6-tagged Get3 with (*Left*) untagged WT Get2-CD or Get2(T34C)-CD and (*Right*) untagged WT Get1-CD or Get1(Q62C)-CD. Controls with no Get3-HIS_6 are shown for comparison. (*C*) Summary of the fluorescence anisotropy end points for complexes of Get1-CD and Get2-CD with the Get3/TA complex in the ADP or apo states. (*D*) Cartoon showing dye placement (cyan) on (*Left*) Get2 T34C (red; Protein Data Bank ID code 3ZS9) and (*Right*) Get1 Q62C (purple; Protein Data Bank ID code 3ZS8) in their respective complexes with the Get3 dimer (yellow). A.U., arbitrary unit.

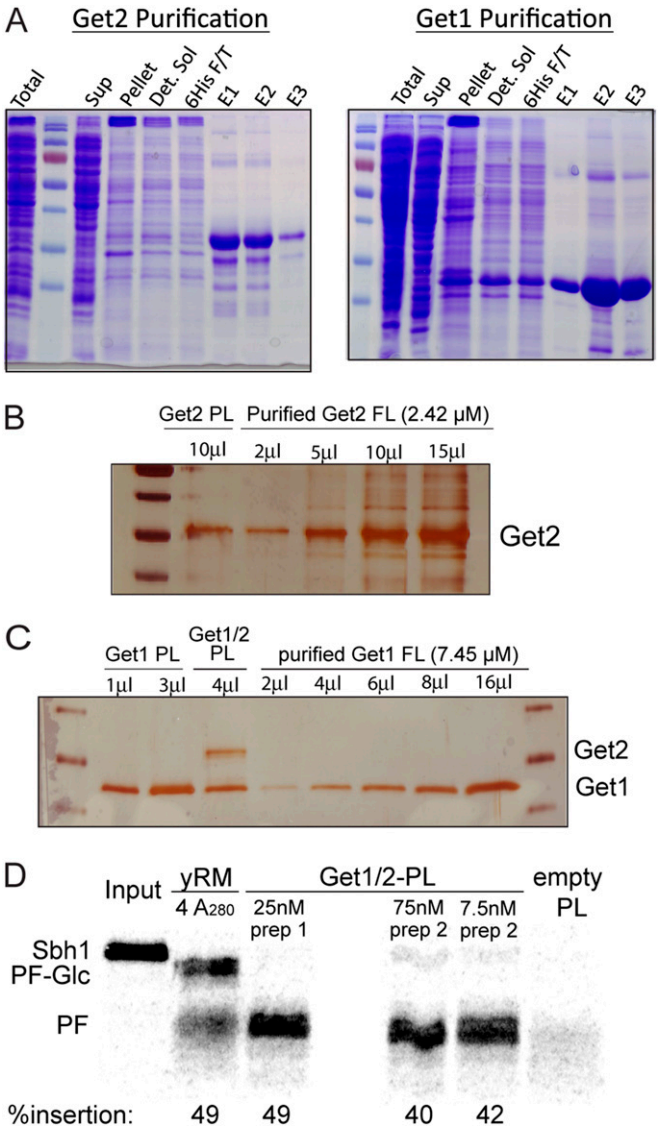


Fig. S4. Reagents for generating Get1/2-PL (related to Fig. 5). (*A*) SDS/PAGE showing the purification of full-length Get1 and Get2 in detergent as described in *Materials and Methods*. (*B*) Silver-stained gel containing Get2-PL and increasing amounts of recombinant Get2. Band intensity of Get2 in PL was quantified using known amounts of recombinant Get2 as a standard for concentration determination. *C* is the same as *B* but with Get1-PL and Get1/2-PL. (*D*) Get3/Sbh1-opsin targeting complexes labeled with ^{35}S-Met were purified from yeast translation extract using an HIS_6 tag on Get3 (*Materials and Methods*) and presented to yRM, Get1/2 PLs, or empty liposomes as indicated. Insertion was analyzed by protection against proteinase K (*Materials and Methods*). Targeting complexes inserted into yRMs also contained a proteinase K-resistant fragment glycosylated to 55% efficiency. Det. Sol, detergent soluble; F/T, flow-through; FL, full-length; Glc, glycosylated; PF, protected fragment; Sup, supernatant.

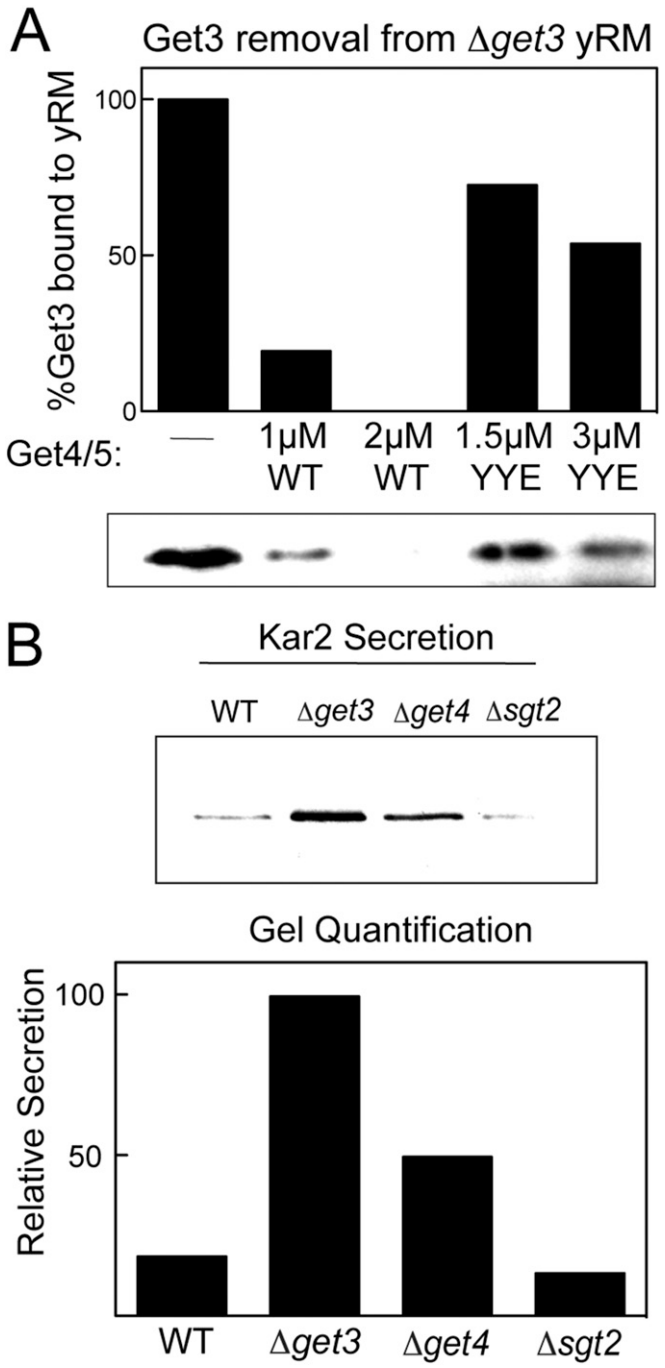


Fig. S5. Get4/5 aids in recycling Get3 from the ER membrane (related to Fig. 7). (*A*) Release of Get3 from yRMs by WT and mutant Get4/5. HIS_6-Get3 was preincubated with yRMs for 15 min in 4 mM ATP and chased with either WT Get4/5 or binding-deficient mutant Get4/5 (YYE). RMs were sedimented as in Fig. 5, and HIS_6-Get3 was detected by Western blot using anti-HIS antibody. (*B*) In vivo assay for TA targeting based on Kar2p secretion (related to Fig. 7). Western blot of secreted Kar2p from the indicated yeast strains (WT, Δ*get3*, Δ*get4*, and Δ*sgt2*) detected it using an anti-Kar2p antibody. *Lower* shows quantification of the Western blot.

Table S1. Summary of the equilibrium binding affinities of the interaction of Get3 with Get4/5

Get4/5 construct	Get3 complex	Nucleotide	K_d (nM)
Full-length Get4/5	Get3	Apo	234 ± 47
		ATP	3.2 ± 2.0
	Get3/TA	Apo	Not detectable
		ATP	46 ± 4
		ADP	49 ± 4
Get4/5N	Get3	Apo	6.0×10^3
		ATP	127 ± 6

Table S2. Summary of the kinetics of the Get3–Get4/5 association in ATP

	First phase		Second phase	
Buffer ionic strength	k_1 (μM^{-1} s^{-1})	Amplitude (%)	k_2 (μM^{-1} s^{-1})	Amplitude (%)
150 mM KOAc	144 ± 12	57	44 ± 5	43
350 mM NaCl	8.9 ± 0.4	64	33 ± 6	36
100 mM NaCl	145 ± 6	59	39 ± 4	41
No salt	272 ± 8	59	39 ± 10	41

Table S3. Summary of the kinetics of Get3 dissociation from Get4/5 in ATP

	First phase		Second phase	
Buffer ionic strength	k_{-1} (s^{-1})	Amplitude (%)	k_{-2} (s^{-1})	Amplitude (%)
150 mM KOAc	0.65 ± 0.12	49	0.10 ± 0.03	51

Table S4. Summary of the equilibrium binding affinities of the interaction of Get3 with Get1-CD and Get2-CD

Receptor	Get3 complex	Nucleotide	K_d (μM)
Get1	Get3	Apo	0.055 ± 0.015
		ADP	0.62 ± 0.19
		ATP	Not detectable
	Get3/TA	Apo	2.50 ± 0.58
		ADP	29.7 ± 4.8
		ATP	Not detectable
Get2	Get3	Apo	0.47 ± 0.09
		ADP	0.44 ± 0.13
		ATP	0.90 ± 0.17
	Get3/TA	Apo	1.61 ± 0.006
		ADP	2.74 ± 0.003
		ATP	2.77 ± 0.82