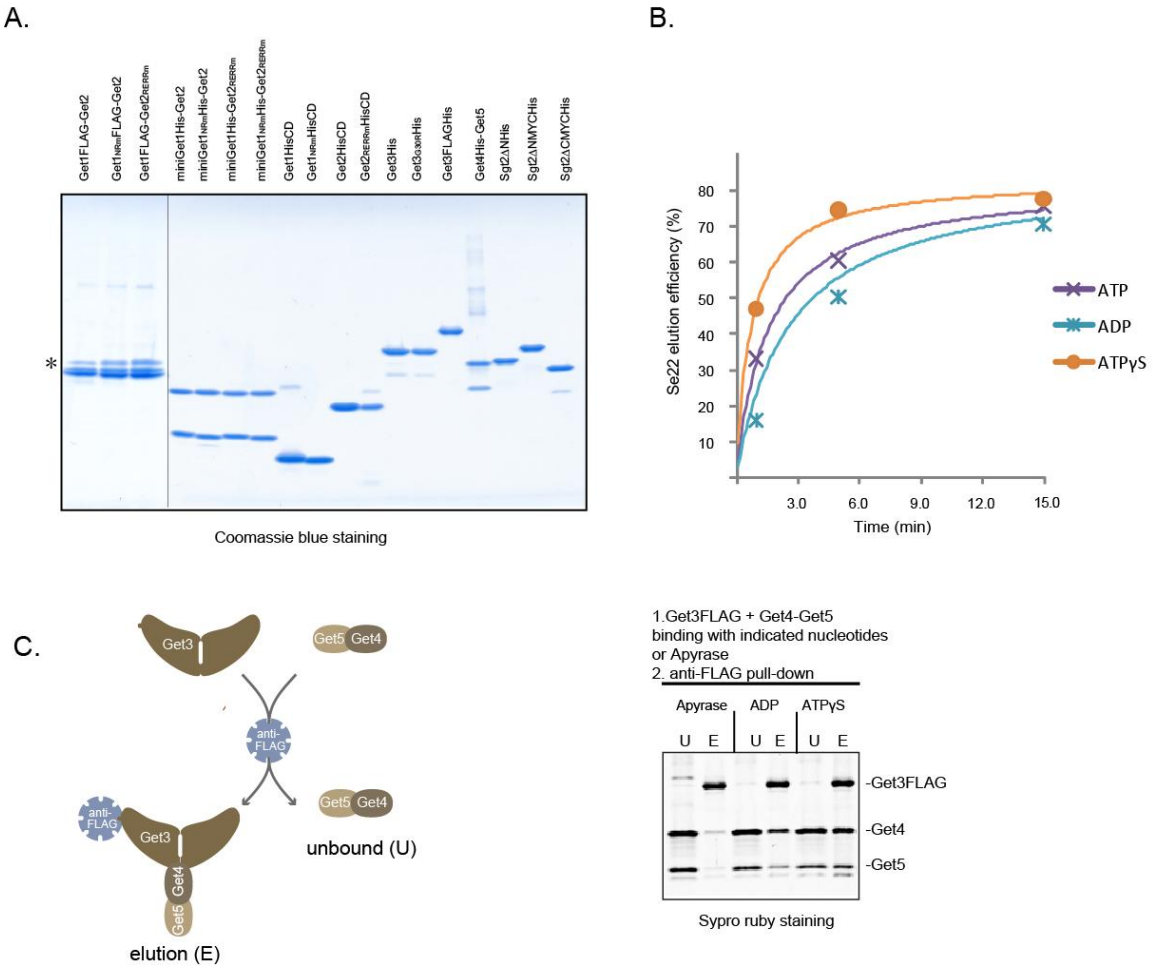
Inventory of Supplemental Information

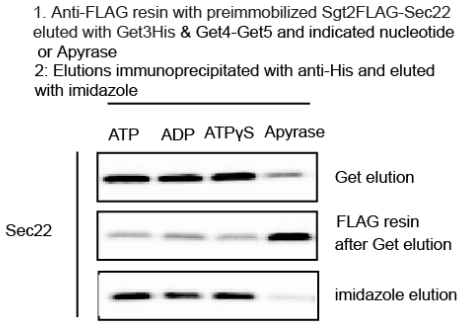
Supplemental Text and Figures

Wang et al. Figure S1, related to Figure 1

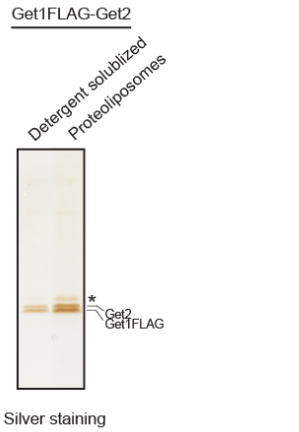


Wang et al Figure S2, related to Figure 2

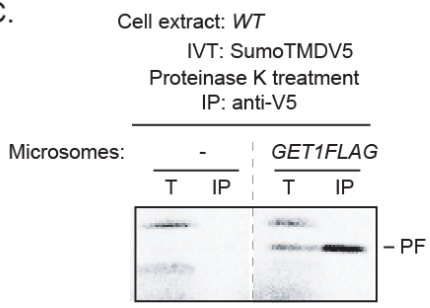
A.



B.



C.



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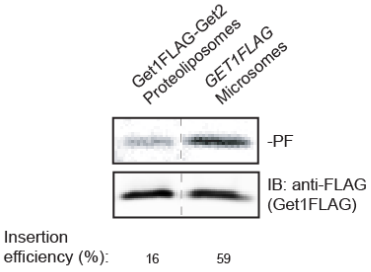
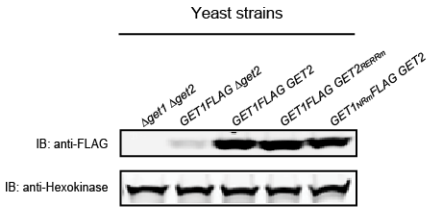
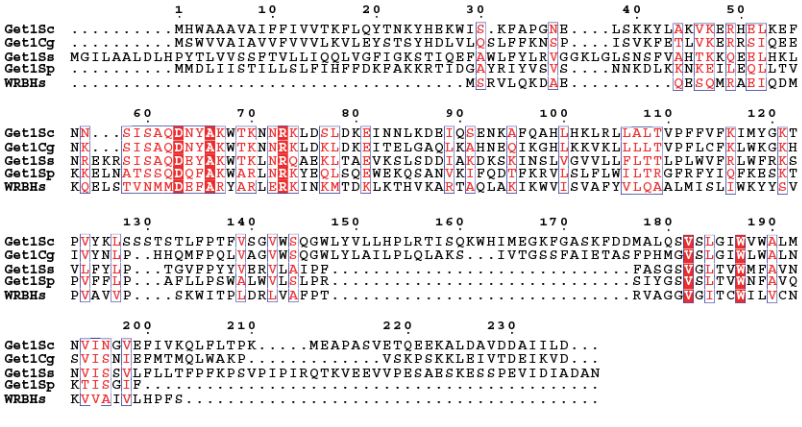


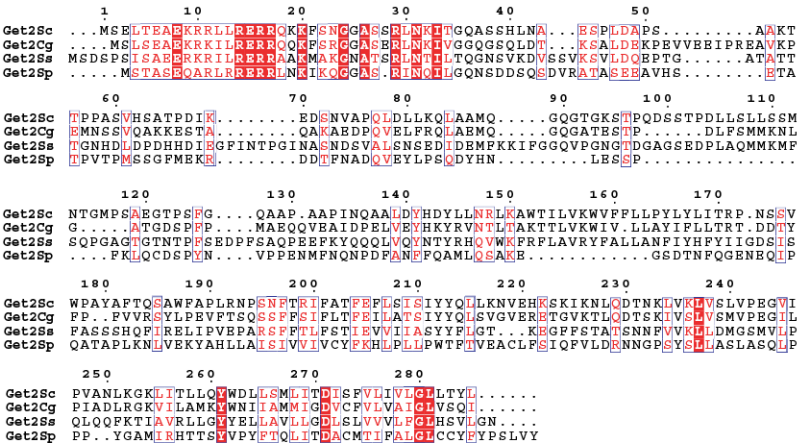
A.

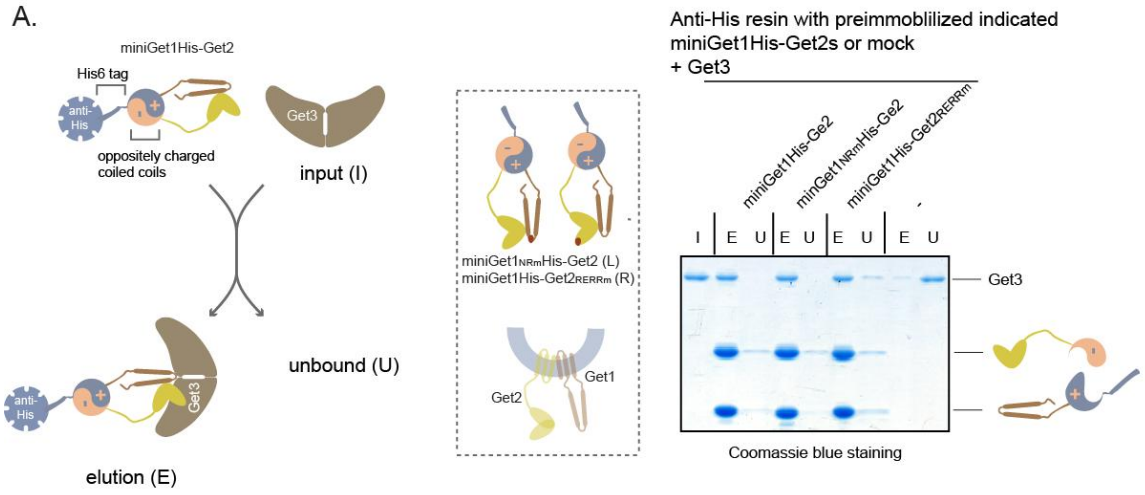

Yeast strains
Δget1 Δget2
GET1FLAG Δget2
GET1FLAG GET2
GET1FLAG GET2REPRH
GET1NRM1FLAG GET2
IB: anti-FLAG
IB: anti-Hexokinase

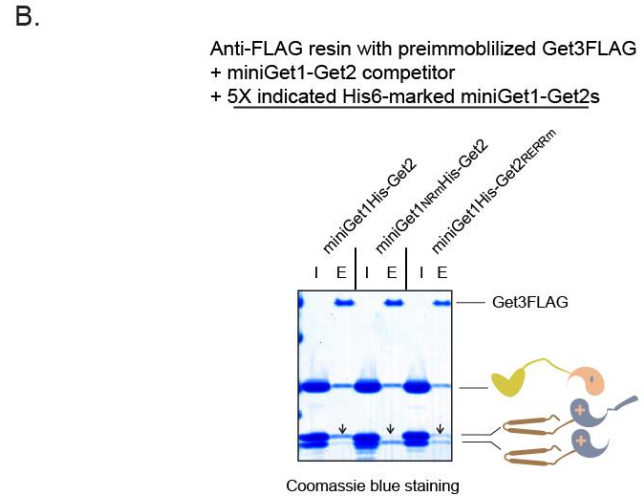
B.


Get1Sc
Get1Cg
Get1Ss
Get1Sp
WRBHs

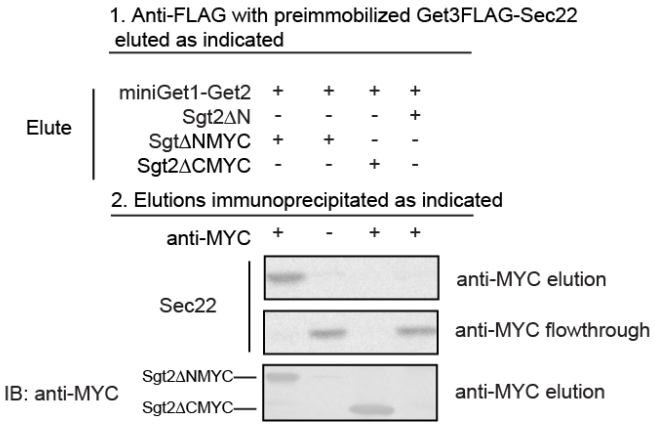
C.


Get2Sc
Get2Cg
Get2Ss
Get2Sp

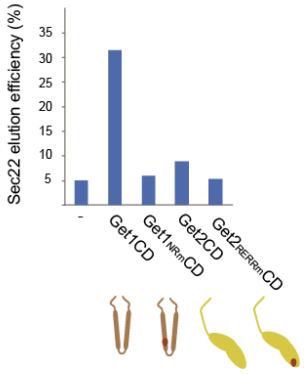

A.
miniGet1His-Get2
His6 tag
anti-His
oppositely charged coiled coils
Get3
input (I)
unbound (U)
elution (E)
miniGet1NRmHis-Get2 (L)
miniGet1His-Get2RERRm (R)
Get1
Get2
Anti-His resin with preimmoblilized indicated miniGet1His-Get2s or mock
+ Get3
miniGet1His-Ge2
miniGet1NRmHis-Ge2
miniGet1His-Get2RERRm
I E U E U E U E U
Get3
Coomassie blue staining


B.
Anti-FLAG resin with preimmoblilized Get3FLAG
+ miniGet1-Get2 competitor
+ 5X indicated His6-marked miniGet1-Get2s
miniGet1His-Get2
miniGet1NRmHis-Get2
miniGet1His-Get2RERRm
I E I E I E
Get3FLAG
Coomassie blue staining

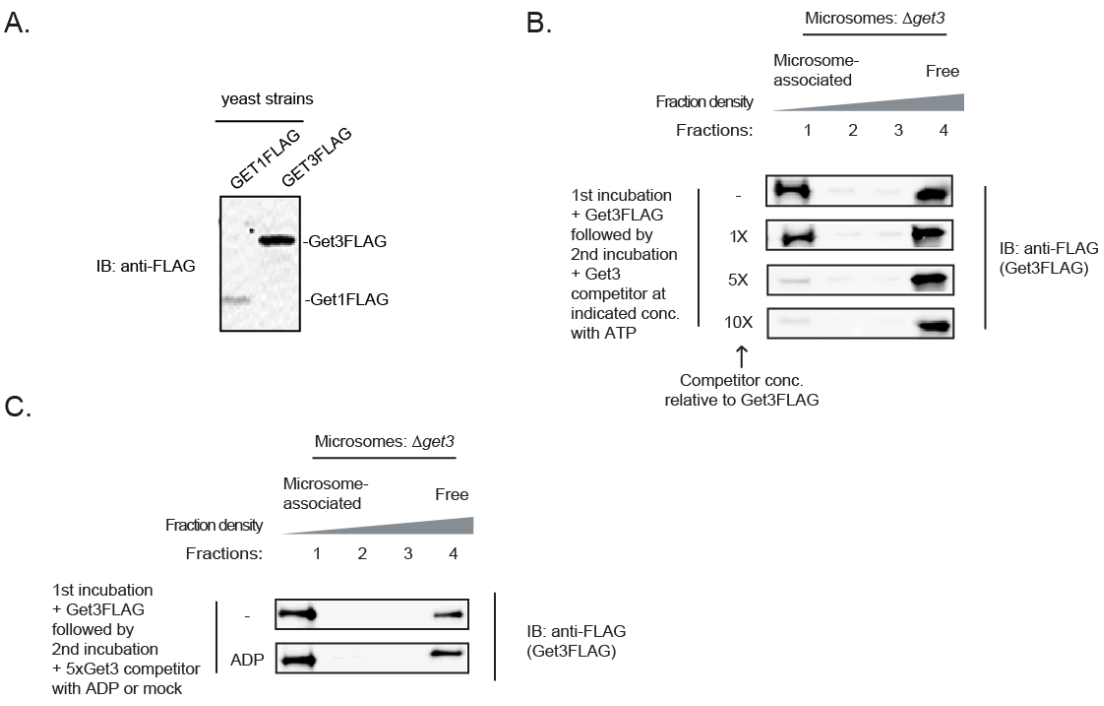
A.


1. Anti-FLAG with preimmobilized Get3FLAG-Sec22 eluted as indicated
Elute
miniGet1-Get2 + + + +
Sgt2ΔN - - - +
SgtΔNMYC + + - -
Sgt2ΔCMYC - - + -
2. Elutions immunoprecipitated as indicated
anti-MYC + - + +
Sec22
anti-MYC elution
anti-MYC flowthrough
IB: anti-MYC
Sgt2ΔNMYC
Sgt2ΔCMYC
anti-MYC elution

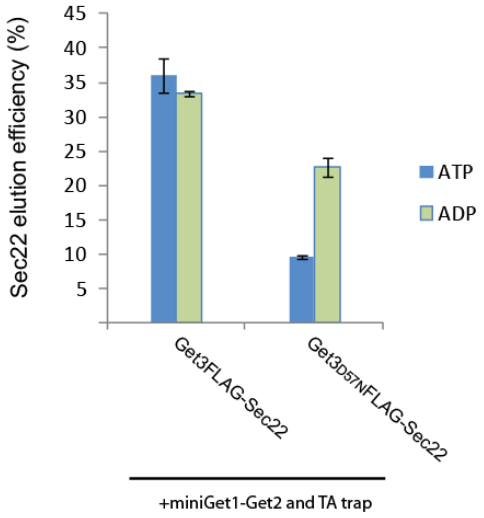
B.


Sec22 elution efficiency (%)
35
30
25
20
15
10
5
-
Get1CD
Get1NRimCD
Get2CD
Get2βERimCD

Wang et al. Figure S6, related to Figure 6



Wang et al. Figure S7, related to Figure 7



SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Purified Proteins Used in this Study; Nucleotide Effects on the Rate of TA Protein Transfer from Sgt2-Get5-Get4 to Get3 and on Get4-Get5 Binding to Get3

(A) The indicated proteins were expressed and purified as described in the Experimental Procedures, resolved by SDS-PAGE, and visualized by Coomassie blue staining. Mass spectrometry of the faint band labeled with * revealed that it is a species of Get2 (data not shown) that most likely results from glycosylation of one of the two predicted luminal N-glycan acceptor sites.

(B) *In vitro* translation (IVT) of Sec22 in *SGT2FLAG* Δ*get3/5* extract was followed by anti-FLAG immunoprecipitation (IP) and elution with Get3 (0.32 μg/μL) and Get4-Get5 (0.48 μg/μL) in the presence of the indicated nucleotides (1 mM). Notably, elution was carried out at 4°C to slow down the kinetics of elution (data not shown) and allow enough time for sample processing. Samples were prepared and analyzed as in Figure 1B. Shown is the percentage of Sec22 eluted at different times following the addition of Get proteins to immobilized Sgt2-Sec22.

(C) (ON THE LEFT) Schematic showing binding of Get4-Get5 to Get3FLAG preimmobilized on anti-FLAG resin. (ON THE RIGHT) Get3FLAG (64 ng/μL) and Get4-Get5 (96 ng/μL) were incubated at room temperature for 20 minutes in the presence of the indicated nucleotides (1 mM). This was followed by anti-FLAG immunoprecipitation at 4°C for 30 minutes. Following centrifugation, the unbound (U) fraction was collected, and the remaining resin washed and eluted with 3XFLAG peptide (E). Samples were resolved by SDS-PAGE and visualized by Sypro ruby staining.

Figure S2. Purification of Get3-Sec22 after Sec22 Transfer from Sgt2-Get5-Get4 to Get3; Purified and Proteoliposome Reconstituted Get1-Get2 Transmembrane Complex; Immunoprecipitation of the PF; Specific Insertion Activity of Proteoliposomes

(A) Sec22 elution from preimmobilized Sgt2-Sec22 with Get3His, Get4-Get5, nucleotides (3 mM) or Apyrase (1 U/μL), as indicated, was carried out as in Figure 1B. Elutions were immunoprecipitated (IP) with anti-His resin and the bound material washed and eluted with imidazole. Samples were resolved by SDS-PAGE and analyzed by autoradiography.

(B) Get1FLAG-Get2 was affinity purified with anti-FLAG resin from digitonin-solubilized microsomes that were obtained from a Δ*get3* strain engineered to overexpress Get1FLAG and Get2. Digitonin was exchanged with BigCHAP prior to elution with 3xFLAG peptide. Proteoliposomes were reconstituted by BigCHAP removal with SM2 Bio-beads in the presence of synthetic phospholipids (see Experimental Procedures for more detail). Purified Get1FLAG-Get2 in BigCHAP and Get1-Get2 proteoliposomes were resolved by SDS-PAGE and visualized by silver staining. Mass spectrometry of the band labeled with * revealed that it is a species of Get2 (data not shown), most likely glycosylated at one of the two N-glycan acceptor sites predicted to reside on the luminal side of the ER membrane.

(C) SumoTMDV5 *in vitro* translated (IVT) in the wild-type (*WT*) extract was incubated with *GET1FLAG* □*get3* microsomes or mock incubated for 30 minutes at room temperature. Following Proteinase K treatment and detergent solubilization, samples were immunoprecipitated (IP) with anti-V5 resin. Eluted and starting total (T) samples were resolved by SDS-PAGE and analyzed by autoradiography. PF indicates the position of the protected fragment.

(D) *GET1FLAG* □*get3* microsomes (1 μL at OD_{280} ~80: an amount comparable to the ones used in all the insertion assays) and Get1FLAG-Get2 proteoliposomes (0.5 μL: an amount comparable to the ones used in all the insertion assays) were analyzed by immunoblotting (IB) with anti-FLAG and protease protection as in Figure 2B.

Figure S3. Total Protein Level of Get1 and Get2 is Unaffected by the NRm/RERRm Mutations; Sequence Alignment of Get1/2 Homologs

(A) Total cell lysates from the indicated yeast strains were resolved by SDS-PAGE and analyzed by immunoblottting (IB) with the indicated antibodies. Note that Get1 levels decrease when Get2 is absent and vice versa (Schuldiner et al., 2008). This suggests that Get1 and Get2 molecules that are not paired up in transmembrane complexes are subject to ER-associated degradation.

(B) and (C) Clustal W2 amino acid sequence alignment of Get1 and Get2 homologs. See Figures 3A and 3B for more details.

Figure S4. Cooperative Binding of Get3 to the Cytosolic Domains of Get1 and Get2

(A) (ON THE LEFT) Schematic showing binding of Get3 to miniGet1His-Get2 preimmobilized on anti-His resin. (ON THE RIGHT) The indicated miniGet1His-Get2s were preimmobilized and incubated with Get3FLAG input (I) (Get3FLAG: miniGet1His-Get2 molar ratio equals 1:6) in the presence of ADP (3mM) for 20 minutes at room temperature. Following centrifugation, the unbound (U) material was collected and the resin was washed and then eluted (E) with SDS gel loading buffer. Samples were resolved by SDS-PAGE and visualized by Coomassie blue staining.

(B) Anti-FLAG resin with pre-immobilized Get3FLAG was incubated with the indicated His-marked miniGet1-Get2s and miniGet1-Get2 competitor inputs (I) (Get3FLAG:miniGet1His-Get2:miniGet1-Get2 molar ratio equals 1:10:2) in the presence of ADP (3 mM) for 20 minutes at room temperature. Following washing, the resin was eluted (E) with FLAG peptide. Samples were resolved by SDS-PAGE and visualized by Coomassie blue staining. To distinguish miniGet1-Get2 from miniGet1His-Get2s on a gel, we have proteolytically removed the C-terminal His6 tag (separated by a thrombin site from the engineered coiled coil fused to Get1) from the former. Arrows point to the positions on the gel at which we expect to see His-tagged Get1 bands if they are able to compete for binding to Get3.

Figure S5. Sec22 Released from Get3-Sec22 by miniGet1-Get2 is in the TA Trap; Get1CD has Substrate-Releasing Activity

(A) Sec22 elution from immobilized Get3FLAG-Sec22 in the presence miniGet1-Get2 (6 μM), the indicated Sgt2 derivatives, and ADP (2.75 mM ADP) for 20 minutes at room temperature. Elutions were immunoprecipitated with protein G Dynabeads with or without the anti-MYC antibody, as indicated. Following centrifugation, the flowthrough was collected and the resin was washed and then eluted with SDS gel loading buffer. Samples were resolved by SDS-PAGE and visualized by immunoblotting (IB) with anti-MYC and autoradiography.

(B) Sec22 elution from immobilized Get3FLAG-Sec22 in the presence of the indicated Get1/2 cytosolic domains (1.5 μM) and nucleotide (2.75 mM). Elution was carried out in the presence of Sgt2ΔN (0.1 μg/μL) and analyzed as in Figure 5B.

Figure S6. Cellular Get3 is in Excess to Get1/2; Excess Get3 Outcompetes Get3FLAG from the Membrane; ADP doesn't Stimulate Get3 Dissociation from the Membrane

(A) Whole cell lysates of *GET1FLAG* and *GET3FLAG* yeast strains were resolved by SDS-PAGE and analyzed by immunoblotting (IB) with anti-FLAG. Anti-hexokinase immunoblotting revealed that the samples were evenly loaded (data not shown). ImageQuant software established that the Get3FLAG signal is approximately six times that of Get1FLAG.

(B) Δ*get3* microsomes (0.8 A_{280} units) were incubated with Get3FLAG (0.1 µg) in the absence of nucleotide for 20 minutes at room temperature (1st incubation), and then split and incubated with ATP (4 mM) and indicated amounts of Get3 competitor (0.1 µg equals 1X, lacking the FLAG tag) for another 20 minutes at room temperature (2nd incubation). Samples were analyzed by flotation analysis and immunoblotting (IB) with anti-FLAG as in Figure 3D.

(C) Δ*get3* microsomes (0.8 A_{280} units) were incubated with Get3FLAG (0.1 µg) in the absence of nucleotide for 20 minutes at room temperature (1st incubation), and then split and incubated with the indicated nucleotides (4 mM) and Get3 competitor (0.5 µg, lacking the FLAG tag) for another 20 minutes at room temperature (2nd incubation). Samples were analyzed by flotation analysis and immunoblotting (IB) with anti-FLAG as in Figure 3D.

Figure S7. MiniGet1-Get2 Can't Induce Substrate Release from Get3D57N in the Presence of ATP but Release is Rescued by ADP

Sec22 elution from immobilized Get3FLAG-Sec22 or Get3$_{D57N}$FLAG by miniGet1-Get2 (6 µM) in the presence of ATP or ADP (both at 2.75 mM). Elution was carried out in the presence of Sgt2ΔN (0.1 µg/µL) and analyzed as in Figure 5B. Average Sec22 elution efficiency and standard deviation from two independent experiments are plotted.

Table 1: *S. cerevisiae* Strains Used in this Study

Genetic Background	Deletion(s)	Epitope Tag	Strain Number
BY4741	*(none)*	*Get3FLAG::kan*	VDY10
		Get1FLAG::kan	VDY9
	Δget1::kan Δget2::nat	(none)	VDY35
	Δget3::kan	(none)	VDY36
	Δget3::ura Δget5::his	*Sgt2FLAG::kan*	VDY47
	Δget3::his	*Get1FLAG::kan*	VDY82
		Get1^{NRm}FLAG::kan	VDY84
		Get1FLAG::kan Get2^{RERRm}	VDY85
		nat::pTDH3 Get1^{NRm}FLAG::kan ura::pTDH3 Get2	VDY168
		nat::pTDH3 Get1FLAG::kan ura::pTDH3 Get2^{RERRm}	VDY173
Obtained from a cross between BY4741 and BY4742	*Δget3::kan*	*nat::pTDH3 Get1FLAG::kan nat::pTDH3 Get2*	VDY343

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

S. cerevisiae Strain Construction

Deletion strains were constructed in the BY4741 genetic background (Brachmann et al., 1998) by PCR-mediated knock-out with one of the following drug resistance or prototrophic marker cassettes: pFA6a-kanMX6/pFA6a-NAT (Longtine et al., 1998), pKlURA/pCgHIS (Goldstein and McCusker, 1999). C-terminal triple FLAG tag and TDH3 promoter were introduced by PCR-mediated epitope/promoter tagging as described previously (Denic and Weissman, 2007).

NRm and RERRm mutations were introduced by first knocking out with *URA3* the appropriate genomic region of either Get1 or Get2, respectively. This was followed by transformation with the appropriate OE PCR products containing the mutations and then 5-FOA counterselection.

VDY343 is a nonparental ditype generated from a cross between
BY4741 *nat::pTDH3Get1FLAG::kan*
and BY4742 *nat::pTDH3Get2 Δget3::kan.*

Plasmid Construction

SumoTMDV5 was created by subcloning annealed complementary oligonucleotides that encode the V5 tag into the NotI/XhoI sites of pET29SUMOTMD (Wang et al., 2010).

The vector for bacterial expression of His6-tagged Get3^{G30R} was created by introducing the G30R mutation into pET29Get3 (Wang et al., 2010) by overlap extension (OE) PCR. Vectors for bacterial expression of His6-tagged Sgt2ΔNMYC, and Sgt2ΔCMYC were created by subcloning annealed complementary oligonucleotides encoding the 2XMYC tag into the BamHI/NotI sites of pET29Sgt2ΔN and pET29Sgt2ΔC (Wang et al., 2010), respectively.

Vectors for bacterial expression of His6-tagged Get1CD and Get2CD were created by subcloning PCR amplified regions of genomic Get1 and Get2 encoding amino acids (a.a.) 19- 102 and 1- 150, respectively, into the NdeI/KpnI restriction sites of pET29b. NRm and RERRm mutations were introduced into pET29Get1CD and pET29Get2CD, respectively, by OE PCR.

The vector for bacterial expression of miniGet1-Get2 was generated by subcloning PCR amplified regions of genomic Get1 (a.a 20-102), coiled coil region of pActPL-Gal4AD (Addgene; amino acid sequence: GSGSLEIEAAFLERENTALETRVAELRQRVQRLRNRVSQNRTRYGPLGGGK), genomic Get2 (a.a. 1-150), and coiled coil region of pActPL-Gal4DBD (Addgene; amino acid sequence: GSGSLEIRAAFLRQRNTALRTEVAELEQEVQRLENEVSQYETRYGPLGGGK) into the NcoI/SacI, SacI/PstI, NdeI/XhoI and XhoI/PacI sites of pETDUET-1 (Novagen), respectively. Notably, only the Get1CD fusion with the coiled coil has a C-terminal His6 tag. After bacterial expression and Ni-NTA purification, Get2CD coiled coil copurifies as

part of the miniGet1-Get2 complex. NRm and RERRm mutations were introduced into pETDUETminGet1-Get2 OE PCR.

All the other vectors used in this study were described previously (Wang et al, 2010 or references therein).

Primer sequences used in these plasmid constructions are available upon request.

Recombinant Protein Expression and Purification

pET-based protein expression was carried out by IPTG induction in BL21 DE3 *E. coli* cells as described previously (Wang et al., 2010). In addition Get3^{G30R}, Get1CD, Get1CD^{NRm}, Get2CD, and Get2CD^{RERRm} were expressed at 17°C for 16 hours to enhance solubility. All the miniGet1-Get2 constructs were expressed at 23 °C for 7 hours. Ni-NTA purification of His-tagged proteins was carried out as described previously (Wang et al., 2010). In addition to the final gel filtration step used in all the purification schemes, some preps necessitated ion-exchange chromatography after elution with imidazole from Ni-NTA to obtain a high level of purity. When appropriate, the His6-tag was removed from purified proteins by Thrombin cleavage as described previously (Wang et al., 2010).

***In Vitro* Transcription, Translation, and TA Protein Insertion**

Capped mRNAs were *in vitro* transcribed from the appropriate PCR products using the mMessage mMachine T7 kit (Ambion) as described previously (Schuldiner et al., 2008).

Yeast *in vitro* translation extracts and translocation-competent, ER-derived membrane (microsomes) were prepared as described before (Wang et al., 2010).

In vitro translation and TA protein insertion were performed as described before (Wang et al., 2010). Insertion efficiency was determined by phosphorimager analysis using a Typhoon imaging system with ImageQuant TL software (GE).

Native Talon (Anti-His) IP

Anti-His IP in experiments shown in Figures S2A, S4A, and 5F was carried out as described previously (Wang et al., 2010).

Protease Protection Assay for Monitoring TA Protein Insertion

This procedure is a modification of the one described previously (Stefanovic and Hegde, 2007). Following insertion, Proteinase K (PK) was added to the final concentration of 0.5 mg/mL and incubated for 1 hour on ice. PMSF was then added to 10 mM and after 5 more minutes on ice, the samples were transferred to 10 volumes of boiling 1% SDS/0.1 M Tris, pH 8. Boiling was continued for at least 2 more minutes to ensure complete inactivation of PK. In the experiments shown in Figures 2B, S2D, and 3D, samples were analyzed directly by SDS-PAGE. In the proof of concept experiment shown in Figure S2C, following PK inactivation, samples were diluted 10-fold with IP buffer (1% Triton X-100, 100 mM NaCl, 50 mM Hepes, pH 7.4) at 4°C and the V5 antibody added along with Protein G Dynabeads (Invitrogen). After overnight (~16 h) incubation at 4°C with agitation, the beads were washed three times with IP buffer and eluted by boiling in SDS-PAGE sample buffer.

Microsome Flotation Analysis

Microsome flotation in an Optiprep gradient was carried out as described previously (Schuldiner et al., 2008), with minor modifications. In the experiment shown in Figure 6B, 10 μL of microsomes were mixed with 1 μL of 0.1 mg/mL recombinant Get3FLAG protein and the final volume was brought up to 24 μL with 20 mM HEPES/KOH, pH 6.8, 5 mM $Mg(OAc)_2$, 150 mM KOAc, and 250 mM sorbitol. After 20 minutes incubation at room temperature, samples were mock incubated without nucleotide on ice for 20 minutes or incubated with 4 mM nucleotide (ATP, ADP or ATPγS) for 20 minutes at room temperature before flotation. In the experiment shown in Figure 3D, 10 μL of Get3FLAG-Sec22 complex purified by native FLAG IP and eluted with 3XFLAG peptide was mixed with microsomes and buffer as described above and incubated for 20 minutes at room temperature before flotation. Gradient fractions were concentrated by chloroform/methanol protein precipitation before SDS-PAGE analysis.

Kar2 Secretion Assay

This assay was performed exactly as described (Belden and Barlowe, 2001) with the following modifications. Samples were precipitated in TCA overnight at -20 °C. Washed acetone pellets were resuspended in 50 μL of alkaline SDS-PAGE loading buffer and one-tenth analyzed by immunoblotting with the anti-Kar2 antibody (gift from P. Walter).

miniGet1-Get2 Competition Pull-Down

1 μg of recombinant Get3FLAGHis was incubated with 10 μL anti-FLAG-M2 affinity gel slurry (Sigma) pre-equilibrated in binding buffer (50 mM HEPES-KOH pH 6.8, 10 mM $Mg(OAc)_2$, 150 mM NaCl, 2mM DTT, 10% glycerol, 3mM ADP) for 30 minutes at 4 °C with agitation. The resin was then washed three times with 200 μL ice-cold binding buffer before incubating it with miniGet1-Get2 proteins as indicated in Figures 4C and S4B (30 μL final volume in binding buffer) for 20 minutes at room temperature. Following three binding buffer washes, bound proteins were eluted with 0.5 mg/mL 3×FLAG peptide (Sigma).

Get3 Pull-Down by MiniGet1-Get2

Dynabeads Talon (Invitrogen) was used according to the manufacturer's instructions. 24 μg of recombinant mini-Get1His-Get2s indicated in Figure S4 were incubated with 10 μL of Dynabeads pre-equilibrated in 100 uL binding buffer (50 mM HEPES-KOH pH 6.8, 10 mM $Mg(OAc)_2$, 150 mM NaCl, 2mM DTT, 10% glycerol, 3mM ATP) for 1 hour at 4 °C with agitation. The resin was then washed three times with 100 μL ice-cold binding buffer before incubating it with 2 μg of Get3 (His Tag removed by Thrombin, 30 μl final volume in binding buffer) for 20 minutes at room temperature. Following three ice-cold binding buffer washes, bound material was eluted with SDS gel loading buffer containing 250 mM imidazole.

Native MYC IP

5 μg of anti-MYC (9E10) antibody was preimmobilized onto 1 mg of Protein G Dynabeads (Invitrogen) according to the manufacturer's instructions. Material eluted

from preimmobilized Get3FLAG-Sec22 in Figure S5A (36 μL) was incubated with Protein G Dynabeads with preimmobilized anti-MYC (or mock) for 30 minutes at 4°C with agitation. Following washing with ice-cold IP buffer (20 mM HEPES-KOH pH 7.4, 2 mM $Mg(OAc)_2$, 100 mM KOAc, 2 mM DTT, 14% glycerol), the resin was eluted with SDS gel loading buffer.

Denaturing Whole-Cell Lysate Preparation

Samples in Figures S3A and S6A were prepared as described previously (Wang et al., 2010).

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