Inventory of Supplemental Information

Figure S1, related to Figure1.

Figure S2, related to Figure2.

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

Supplemental Experimental Procedures.

Supplemental Text and Figures

Wang et al. Figure S1, related to Figure 1

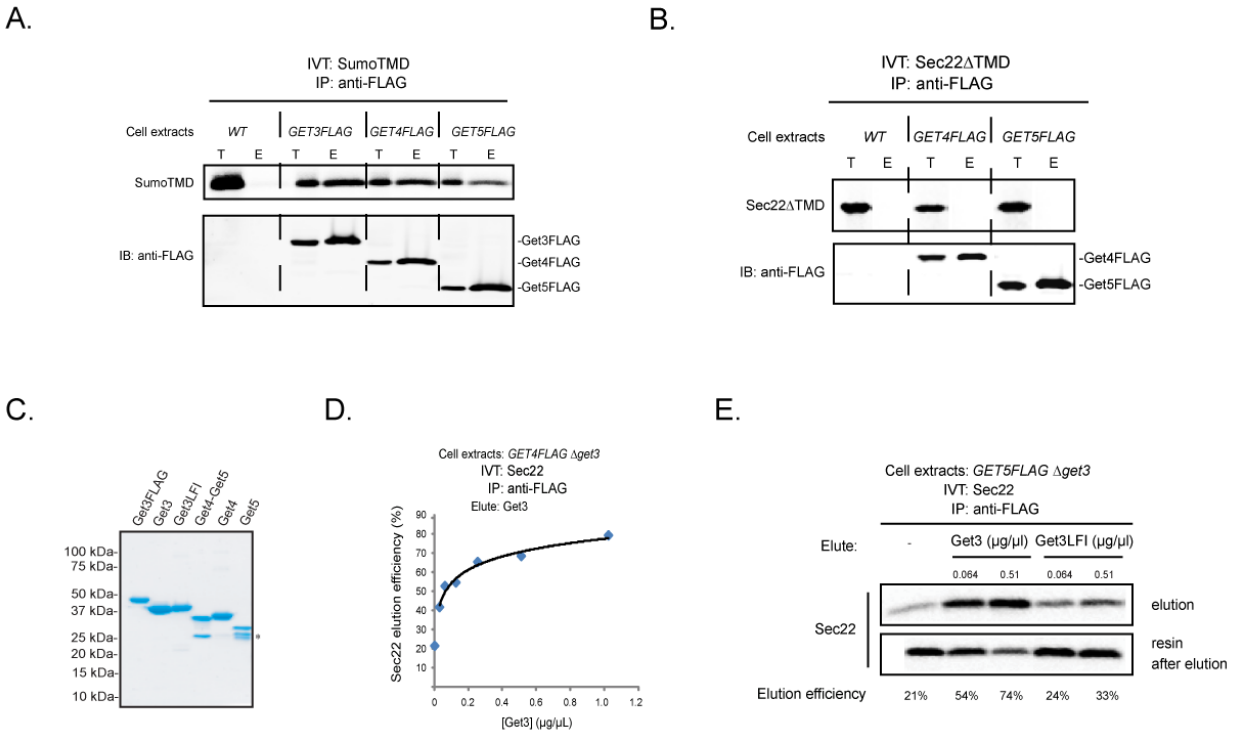


Figure S1.

(A) and (B) *In vitro* translation (IVT) of Sumo fused to Sec22's TMD (SumoTMD) or Sec22 lacking the TMD (Sec22ΔTMD) in the indicated wild type (*WT)* or FLAG tagged extracts was followed by anti-FLAG immunoprecipitation (IP) and 3xFLAG peptide elution. 1.5% of total (T) and 30% of elution (E) were resolved by SDS-PAGE and analyzed by autoradiography and immunoblotting (IB).
(C) The indicated proteins were prepared from *E.coli*, as detailed in the Experimental Procedures for recombinant protein expression and purification, and analyzed by SDS-PAGE followed by Coomassie blue staining. Note that the Get5 band in the Get4-Get5 preparation is untagged and thus migrates faster than the corresponding His-tagged Get5 band in the Get5 preparation.
* indicates the positions of two co-purifying Get5 degradation products in the Get5 preparation (data not shown).
(D) *In vitro* translation (IVT) of Sec22 in *GET4FLAG Δget3* extracts was followed by anti-FLAG immunoprecipitation (IP) and elution with different concentrations of Get3 for 20 minutes at room temperature. Shown is the percentage of Sec22 eluted at different Get3 concentrations (starting at 32 ng/μl). Note that the background elution following mock-treatment was not used in the curve fitting. A separate hyperbolic fitting (which included the background elution, see Experimental Procedures) yielded a half-maximal Get3 concentration of 0.07μg/μL.
(E) *In vitro* translation (IVT) of Sec22 in *GET5FLAG Δget3* extract was followed by anti-FLAG immunoprecipitation (IP) and elution with indicated concentrations of Get3 or Get3LFI for 20 minutes at room temperature or mock treatment. Following centrifugation, elutions were collected, the resin was washed and eluted with gel loading buffer (resin after elution). Both elutions were resolved by SDS-PAGE and analyzed by autoradiography. Shown at the bottom is the percentage of total Sec22 eluted by Get3.

Wang et al. Figure S2, related to Figure 2

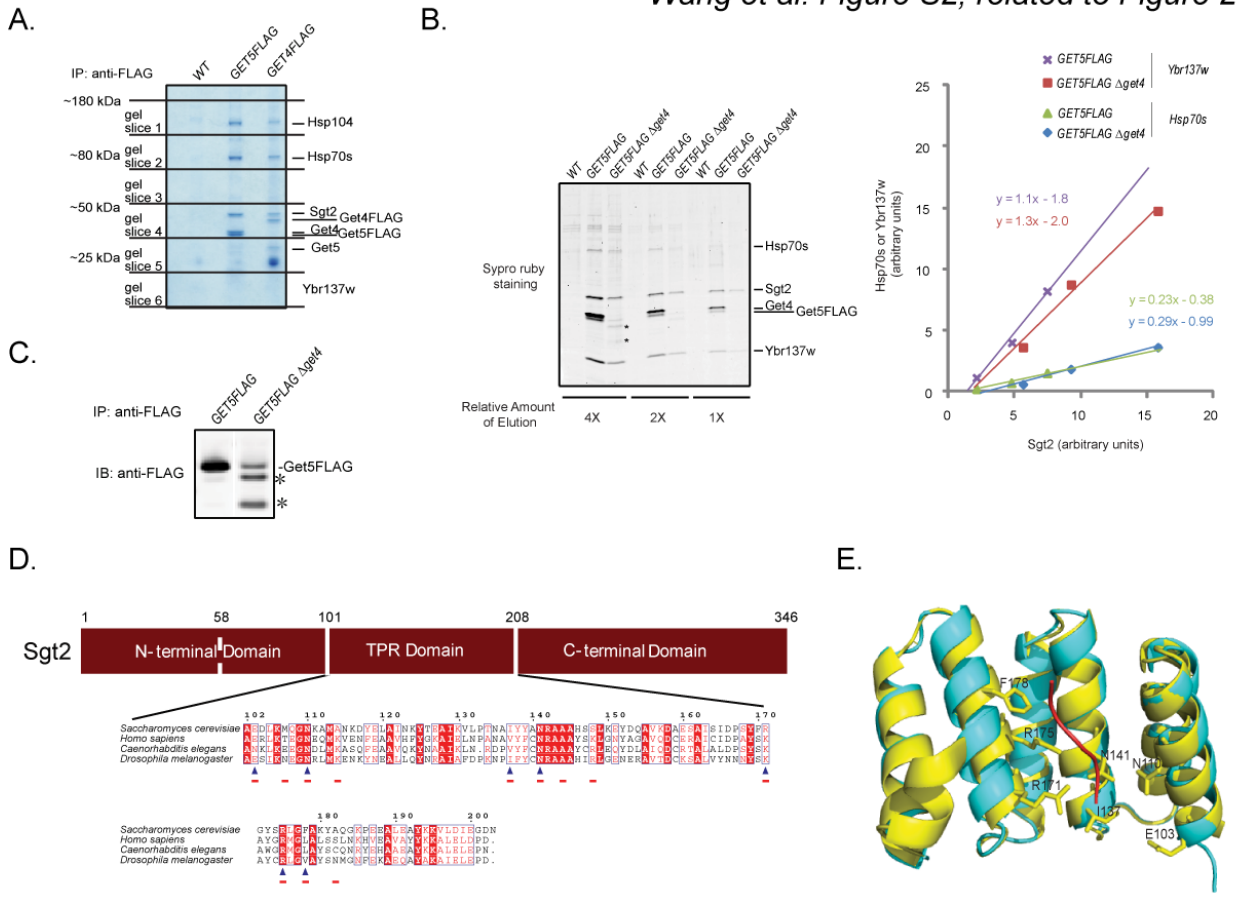


Figure S2.

(A) Anti-FLAG resin was incubated with cytosolic lysates from the wild type (*WT*) and indicated FLAG-tagged strains, washed, and eluted with 3xFLAG peptide. Eluted proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining. Each lane was cut into six slices, as indicated, and separately analyzed by mass spectrometry. Get4, Get5, Hsp104, Hsp70s, Sgt2, and Yrb137w had log2>4 enrichment scores in both FLAG-tagged preparations. Note that Ybr137w can't be clearly seen in gel slice 6 (see Ybr137w band in Figures2A and S2D). Detailed peptide count/sequence information is available upon request.
(B) Cytosolic lysates from the indicated FLAG-tagged strains were immunoprecipitated (IP) with anti-FLAG resin. Following washing, the resin was eluted with 3xFLAG peptide. The indicated relative amounts of eluted proteins were resolved by SDS-PAGE and visualized by Sypro ruby staining. * indicate two partial proteolysis products of Get5FLAG in the Δget4 preparation (see also Figure S2C). Shown on the right is a quantitative analysis of the gel on the left presented as the Sypro ruby signals (in arbitrary fluorescence units determined by ImageQuant analysis) for either Hsp70s or Ybr137w plotted against the corresponding Sgt2 signals. The data were fit to a straight line by linear regression analysis.
(C) The indicated elutions from Figure 2A were resolved by SDS-PAGE and immunoblotted (IB). * indicates two prominent Get5FLAG degradation products.
(D) Schematic of the three protein domains of Sgt2. Shown below is the Clustal W2 amino acid sequence alignment of the TPR domains of Sgt2 homologs from *S. cerevisiae* (NP_014649.1), *Homo sapiens* (NP_003012.1), *Caenorhabditis elegans* (NP_494893.1), and *Drosophila melanogaster* (NP_609842.1). ESPript 2.0 was used to highlight identical (white in color boxed with red), and well-conserved (red in color boxed in white) residues. Red dashes indicate the positions predicted to face the ligand-binding groove. Blue triangles indicate the positions mutated to alanines in S2D.
(E) A PyMOL cartoon representation of the homology model of *S. cerevisiae* Sgt2 residues 101 to 207 (yellow) made by Rosetta Comparative Modeling (Chivan, D. and Baker, D., 2006) superimposed on the cartoon of the crystal structure of CHIP ubiquitin ligase TRP domain (cyan) bound to the Hsp90 C-terminal peptide (red) (PDB: 3KD7). Sgt2 residues mutated in this study are indicated as sticks.

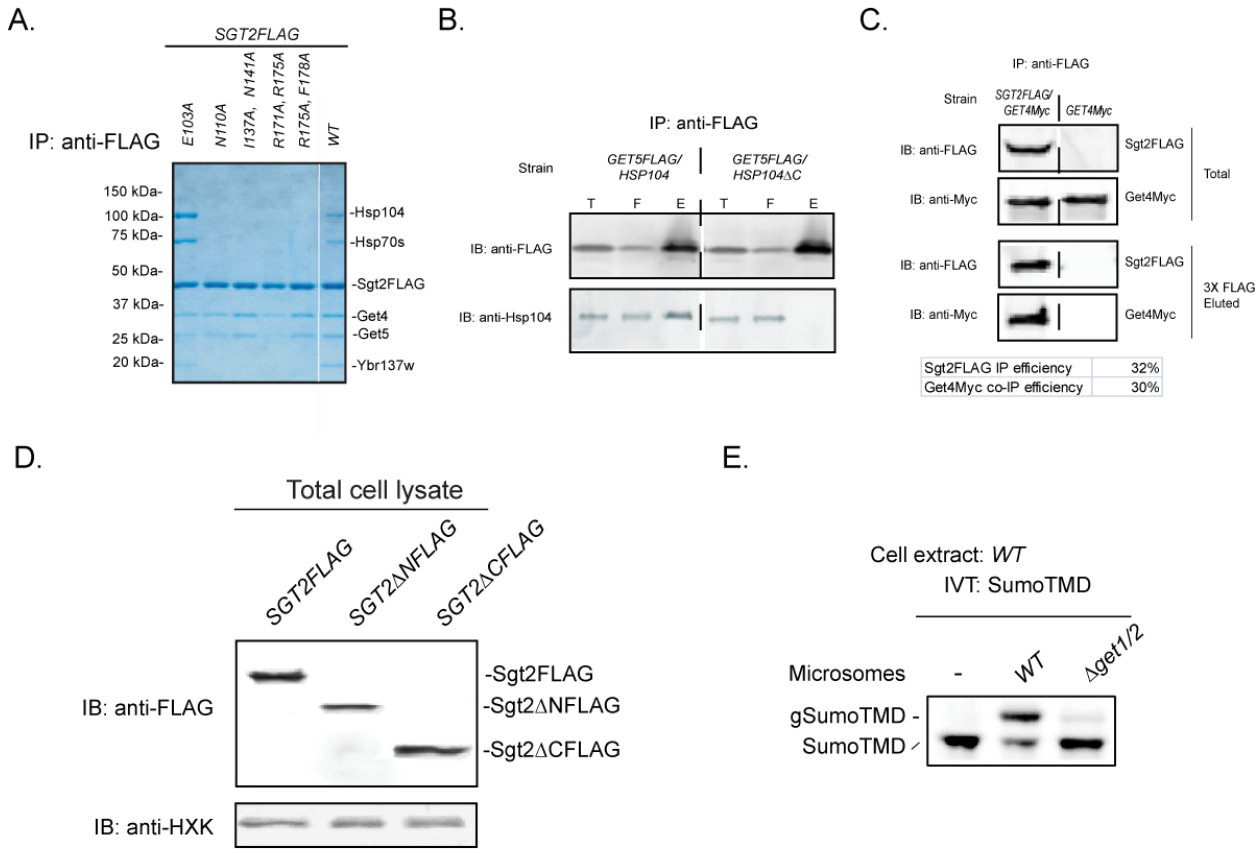


Figure S3.

(A) Anti-FLAG resin was incubated with cytosolic lysates from FLAG-tagged Sgt2 wild type (*WT*) and indicated TPR point mutant strains, washed, and eluted with 3xFLAG peptide. Eluted proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining.
(B) Cytosolic lysates from the indicated FLAG-tagged strains were immunoprecipitated (IP) with anti-FLAG resin. The IP input (T) (4%), flowthrough (F) (4%), and material eluted with 3xFLAG peptide from the washed resin (E) (100%) were resolved by SDS-PAGE and analyzed by immunoblotting (IB) with the indicated antibodies.
(C) Cytosolic lysates from the indicated strains were immunoprecipitated (IP) with anti-FLAG resin. Following washing, the resin was eluted with 3xFLAG peptide. The starting (Total) and eluted (3X FLAG Eluted) material was resolved by SDS-PAGE and analyzed by immunoblotting (IB) with the indicated antibodies. To facilitate Western signal quantitation, we used Typhoon imaging with simultaneous two-color detection of fluorescent secondary antibodies. Shown below is the percentage of total cellular Sgt2FLAG and Get4Myc present in the eluted material.
(D) Δget3 cells expressing FLAG-tagged full length Sgt2 and the indicated truncations were grown to mid-log phase and lysed in gel loading buffer. Following centrifugation, samples were resolved by SDS- PAGE and analyzed by immunobloting (IB) with anti-FLAG and anti-Hexokinase (HXK).
(E) *In vitro* translation (IVT) of SumoTMD in wild type (*WT*) extracts was followed by incubation with WT or Δget1/2 microsomes for 30 minutes at room temperature or mock treatment. Samples were resolved by SDS-PAGE and analyzed by autoradiography. The positions of SumoTMD and glycosylated SumoTMD (gSumoTMD) are indicated. A carboxyl-terminal Opsin tag with an N-glycan acceptor site was used to monitor insertion (see Figure 1E).

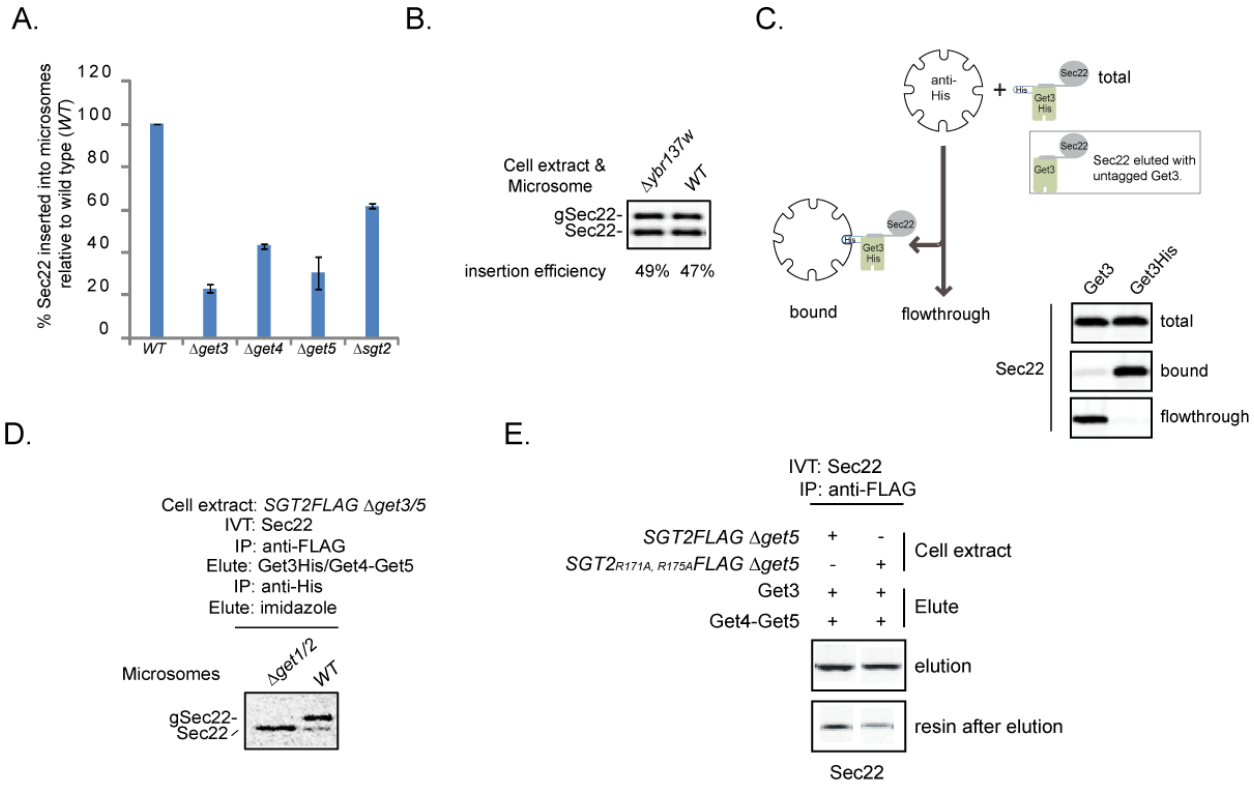
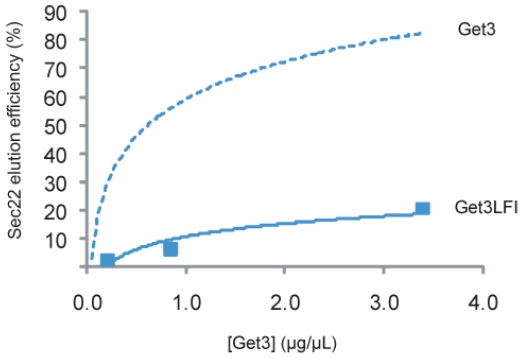


Figure S4.

(A) *In vitro* translation (IVT) of Sec22 in wild type (*WT*) and indicated deletion extracts was followed by incubation with genetically matching microsomes for 30 minutes at room temperature. Shown is the average and standard deviation (three independent experiments) of Sec22 insertion efficiency in each extract relative to wild type.
(B) *In vitro* translation of Sec22 in wild type (*WT*) and Δybr137w extracts was followed by incubation with genetically matching microsomes for 20 minutes at room temperature. A carboxyl-terminal Opsin tag with an N-glycan acceptor site was used to monitor insertion (see Figure 1E). The positions of Sec22 and glycosylated Sec22 (gSec22) are indicated.
(C) IVT of Sec22 in SGT2FLAG Δget3/5 extract was followed by anti-FLAG immunoprecipitation (IP) and elution with Get3His/Get4-Get5 or Get3 (i.e., His tag removed by thrombin cleavage, see Experimental Procedures for details)/ Get4-Get5 (35 ng/μL Get3His/Get3 and 70 ng/μL Get4-Get5) for 20 minutes at room temperature. Elutions were then incubated with anti-His resin, washed, and eluted with imidazole, as shown in the accompanying schematic. 15% each of total, imidazole elution, and flowthrough were resolved by SDS-PAGE and analyzed by autoradiography.
(D) The indicated imidazole elution from Figure S5B was incubated with wild type (*WT*) or Δget1/2 microsomes for 30 minutes at room temperature. Samples were resolved by SDS-PAGE and analyzed by autoradiography. The positions of Sec22 and glycosylated Sec22 (gSec22) are indicated (see Figure 1E).
(E) IVT of Sec22 in the indicated extracts was followed by anti-FLAG IP and elution with Get3 (64 ng/μl) and Get4-Get5 (130 ng/μl) for 20 minutes at room temperature. Samples were prepared and analyzed as in Figure 5B.

A.



B.

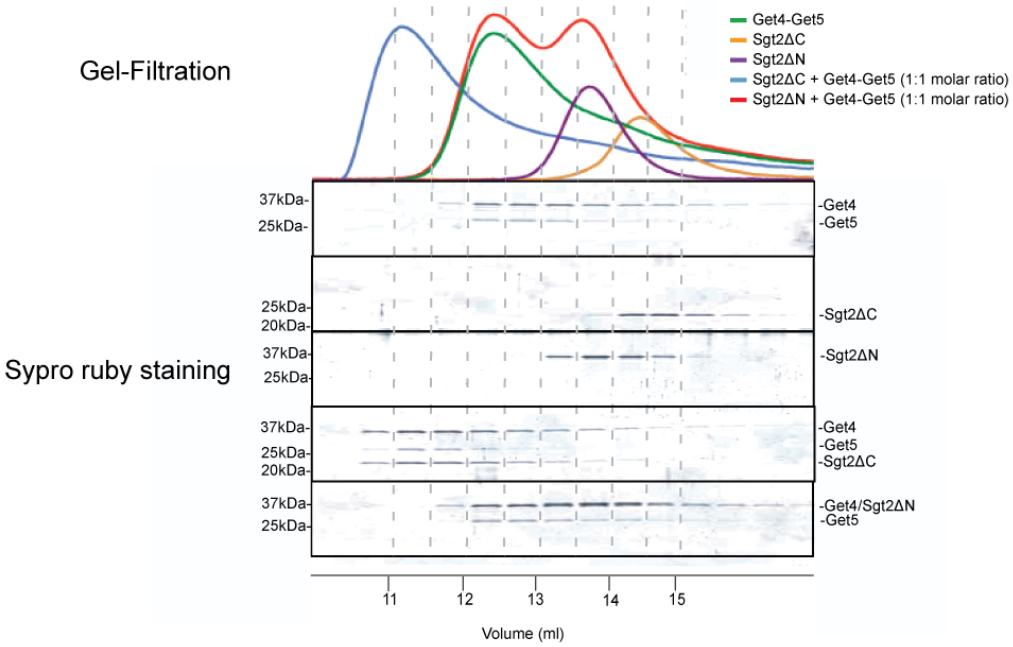


Figure S5.

(A) *In vitro* translation (IVT) of Sec22 in *SGT2FLAG Δget3/5* extract was followed by anti-FLAG immunoprecipitation (IP) and elution with indicated concentrations of Get3LFI for 20 minutes at room temperature. Shown is the precentage of Sec22 eluted at different concentration of Get3LFI and a reference wild type Get3 curve from Figure 6A.

(B) The indicated recombinant proteins were analyzed by gel-filtration chromatography (Superdex 200 10/300 GL). The relevant fractions (indicated by dash lines) were resolved by SDS-PAGE and visualized by Sypro ruby staining. Shown above and below are the A_{280} traces and elution volumes, respectively, corresponding to the fractions analyzed.

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

Genetic Background	Deletion(s)	Epitope Tag	Strain Number
BY4741	*Δget1::kan* *Δget2::nat*	(none)	VDY35
	Δget3::kan	(none)	VDY36
		Get4FLAG::kan	VDY37
		Get5FLAG::kan	VDY38
	Δget3::ura	*Sgt2FLAG::kan*	VDY39
	Δget4::his	(none)	VDY40
		Get3FLAG::kan	VDY41
		Get5FLAG::kan	VDY14
	Δget5::ura	(none)	VDY42
		Get3FLAG::kan	VDY43
		Get4FLAG::nat	VDY18
	Δget5::his	*Sgt2FLAG::kan*	VDY44
		$Sgt2_{R171A,\ R175A}$*FLAG::kan*	VDY45
	Δsgt2::his	(none)	VDY25
		Get5FLAG::kan	VDY12
	Δget3::ura *Δget5::his*	*Sgt2FLAG::kan*	VDY47
	(none)	*Get3FLAG::kan*	VDY10
		Get4FLAG::kan	VDY49

		Get5FLAG::kan	VDY50
		Sgt2FLAG::kan	VDY15
		Get6$_{R171A, R175A}$FLAG::kan	VDY51
		Sgt2$_{E103A}$FLAG::kan	VDY52
		Sgt2$_{N110A}$FLAG::kan	VDY53
		Sgt2$_{I137A, N141A}$FLAG::kan	VDY54
		Sgt2$_{R175A, F178A}$FLAG::kan	VDY55
		HSP104ΔC::nat,Get5FLAG::kan	VDY46
		HSP104::nat, Get5FLAG::kan	VDY48
		Get4Myc13::his	VDY112
		Get4Myc13::his *Sgt2FLAG::kan*	VDY113
		Get5Myc13::his	VDY114
		Get5Myc13::his *Sgt2FLAG::kan*	VDY115
BY4742	*Δget3::kan*	(none)	VDY56
		Sgt2FLAG::nat	VDY57
		Sgt2ΔNFLAG::nat	VDY58
		Sgt2ΔCFLAG::nat	VDY59

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mass Spectrometry Analysis

Lanes indicated in Figure S2A were reduced, carboxyamidomethylated and digested with trypsin. Peptide sequence analysis of each digestion mixture was performed by microcapillary reversed-phase high-performance liquid chromatography coupled with nanoelectrospray tandem mass spectrometry (μLC-MS/MS) on an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, MA). The Orbitrap repetitively surveyed an m/z range from 395 to 1600, while data-dependent MS/MS spectra on the ten most abundant ions in each survey scan were acquired in the linear ion trap. MS/MS spectra were acquired with relative collision energy of 30%, 2.5-Da isolation width, and recurring ions dynamically excluded for 60 s. Preliminary sequencing of peptides was facilitated with the SEQUEST algorithm with a 30 ppm mass tolerance against the yeast proteome of the UniProt Knowledgebase. With a custom version of Proteomics Browser Suite (ThermoFisher Scientific, San Jose CA), peptides were accepted with mass error <2.5ppm and score thresholds to attain an estimated false discovery rate of ~1% using a reverse decoy database strategy. Spectral counts were used to compare proteins across different states/preparations.

Denaturing Whole-Cell Lysate Preparation

1 OD_{600} unit of yeast cells grown in YPD at 30°C to mid-log phase (OD_{600} ~0.7) was harvested by centrifugation for 2 minutes at 21,000 rcf. Cell pellets were washed with 500 μl water and centrifuged again. Washed pellets were frozen at -80 °C for 20 minutes and lysed in 100 μl SDS-PAGE sample buffer containing 1× Roche complete protease inhibitor cocktail at 65 °C for 20 minutes. Following centrifugation for 10 minutes at 21,000 rcf, samples were resolved by SDS-PAGE and analyzed by immunoblotting.

Native Talon (Anti-His) IP

Dynabeads Talon (Invitrogen) were used according to the manufacturer's instructions. Briefly, 15 μl of Sec22 elutions in Figure S5B was mixed with 20 μl (1 mg) of beads equilibrated in binding/washing buffer (20 mM HEPES-KOH pH 7.4, 2 mM $Mg(OAc)_2$, 100 mM KOAc, 2 mM DTT, 14% Glycerol) at 4 °C for 30 minutes with agitation. Following washing with binding/washing buffer, bound proteins were eluted with 30 μl binding/washing buffer with 250mM imidazole for 15 minutes at room temperature with agitation. To remove imidazole, elutions were applied to an Illustra probeQuant G-50 micro column (GE Healthcare) pre-equilibrated in binding/washing buffer.

Gel-Filtration Complex Analysis

The indicated proteins in Figure S6B were incubated at 9 μM each in a total volume of 200 μl SEC buffer (50 mM HEPES-NaOH pH 6.8, 150 mM NaCl, 2% glycerol and 2mM β-mercaptoethanol) at 4° C for 30 minutes with agitation. Following centrifugation for 10 minutes at 21,000 rcf and 4° C, samples were injected onto a GE Superdex 200 10/300 GL size exclusion column pre-equilibrated in SEC buffer. Eluted fractions were resolved by SDS-PAGE, visualized by Sypro Ruby (Invitrogen) staining, and analyzed using a Typhoon imaging system with ImageQuant TL (GE) software.