Supporting Information

Heck et al. 10.1073/pnas.0910591107

SI Text

Strains and Plasmids. Yeast strains were cultured, as described elsewhere (1), in minimal media with 2% (vol/vol) dextrose and appropriate amino acid supplements at 30°C unless otherwise indicated. The majority of strains used were in the BY4741 background (*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*), with the exception of *ubc4Δubc5Δ* and WT, MHY508 (*ubc4Δ::HIS3 ubc5Δ::LEU2*), MHY501 (*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) with the exception of: *ubc4Δubc5Δ* and WT, MHY508 (*ubc4Δ::HIS3 ubc5Δ::LEU2*), and MHY501 (*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1–1*). *SSA1* (JN516; *MATα ura3-52 leu2-3 his3-11, 15 trp1-Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*) and *ssa1–45* (JB67; *MATα ura3-52 leu2-3 his3–11, trp1-Δ1 lys2 ssa1::ssa1–45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*). Null alleles with coding regions replaced were constructed in the BY4741 background by transforming yeast using the Lithium Acetate method with a PCR product encoding the indicated selection marker and 50-bp flanks homologous to the gene to be disrupted (2) or using knockout cassettes in the laboratory collection. Oligo sequences are available on request.

The *UBR1* (pRH2444), *UBR1MR1* (pRH2445), and *PADH-UBR1* (pRH2471) plasmids were a gift from A. Varshavsky (California Institute of Technology, Pasadena, CA). The original -CPY*-*GFP* expression plasmid was provided by D. Wolf (University of Stuttgart, Stuttgart, Germany). The *SAN1* (pRH2475), *SAN1-NLS* (pRH2439), *san1Δ::NatMX* (pRH2376), *PGAL-CPY*-GFP* (pRH2533), and *PGALCPY*- GFP-NES* (pRH2534) plasmids were a gift from R. Gardner (University of Washington, Seattle, WA).

Degradation Assays. Cycloheximide chase degradation assays were performed as previously described (1). Briefly, yeast cells were grown to log phase (~OD_{600} < 0.5), and cycloheximide was added to a final concentration of 50 μg/mL. At the indicated time points, cells were collected by centrifugation and lysed with 0.1 mL of SUME [1% SDS, 8 M urea, 10 mM Mops (pH 6.8), 10 mM EDTA] with protease inhibitors (260 μM ABESF, 142 μM TPCK, 100 μM leupeptin, 76 μM pepstatin) and 0.5-mm glass beads, followed by vortexing for 2 min and addition of 100 μL of 2× USB [75 mM Mops (pH 6.8), 4% (vol/vol) SDS, 200 mM DTT, 0.2 mg/mL bromophenol blue, 8 M urea]. The bead slurry was heated to 80°C for 3 min and then clarified by centrifugation before separation by SDS/PAGE and subsequent immunoblotting with appropriate antibodies.

Flow Cytometry Analysis. Flow cytometry for GFP-tagged substrates was performed as described elsewhere (3). Cell cultures were grown in minimal medium to low log phase (OD_{600} = 0.1) before addition of 50 μg/mL cycloheximide for the indicated times. Samples were measured for fluorescence with a BD Biosciences FACScalibur instrument, and statistical analysis was conducted with CellQuest flow cytometry software. Histograms represent 10,000 individual cells.

Cytoplasm Preparation. Cytoplasm for in vitro assays was prepared from the respective genetic backgrounds using an approach modified from our in vitro ERAD assay (4). Cells were grown in yeast peptone dextrose (YPD) to an OD_{600} of 0.8–1.0, and 100 ODs of cells were pelleted. The pellet was washed twice with H_2O and once with cold B88 buffer [20 mM Hepes-KOH (pH 7.4), 150 mM KOAc, 250 mM sorbitol, 5 mM Mg(OAc)2] with protease inhibitors (260 μM ABESF, 142 μM TPCK, 100 μM leupeptin, 76 μM pepstatin) and DTT, and it was resuspended in 100 μL of B88 buffer with protease inhibitors and DTT for lysis by grinding in a mortar and pestle. The mortar and pestle were precooled with liquid nitrogen before addition of the cells. The cells were added to the mortar with 5 mL of liquid nitrogen. The frozen cells were ground by hand with the pestle. The cells were kept frozen during the process by addition of liquid nitrogen as needed. The ground cells were then placed in a 2-mL tube on ice and allowed to thaw back to liquid. The resulting cytoplasm was clarified by centrifugation at 5,000 × *g* at 4°C for 5 min. The supernatant was then transferred to a different tube and centrifuged again at 20,000 × *g* at 4°C for 15 min. A final ultracentrifugation was carried out at 100,000 × *g* at 4°C for 60 min. Protein concentration of individual cytoplasms was determined using Bradford Reagent (Sigma-Aldrich). Cytoplasms were kept on ice until use.

In Vitro Ubiquitination Assay. Bead-bound immunoprecipitated substrate was mixed with the isolated cytoplasm from the indicated genetic background in the following way: All cytoplasmic reactions took place in a final volume of 30 μL and were prepared on ice. One hundred fifty milligrams of total protein from the respective cytoplasmic preparation was mixed with 15 mM ATP and 10 μL of FLAG beads bound to pre-IP substrate. The reactions were incubated in a 30°C water bath for 1 h with periodic agitation. The reaction was terminated by adding 800 μL of IP buffer (15 mM sodium phosphate, 150 mM NaCL, 10 mM EDTA, 2% (vol/vol) Triton X-100, 0.1% (vol/vol) SDS, 0.5% (vol/vol) deoxycholate) with protease inhibitors and 5 mM *N*-ethylmaleimide. The FLAG beads were washed three times with 1 mL of IP wash buffer (50 mM NaCl, 10 mM Tris, pH 7.5), aspirated to dryness, and heated in the presence of sample buffer to 100°C for 3 min before SDS/PAGE and immunoblotting. IP of substrate before in vitro ubiquitination experiments was conducted in the following manner. Strains lacking San1 and Ubr1 and containing FLAG-tGND1-GFP were grown as described for the previous cytoplasmic preparation. Ten microliters of anti-FLAG M2 beads (Sigma Aldrich) was added per 150 mg of cytosol and allowed to nutate overnight at 4°C. The beads were then pelleted in an Eppendorf 5415c microfuge at 1,000 rpm for 1 min. Three washes with IP buffer were conducted before final resuspension in B88 reaction buffer.

In Vivo Ubiquitination Assay. Cells were grown and lysed as outlined above. To assess in vivo ubiquitination, 1 mL of IP buffer with protease inhibitors and N-ethylmaleimide was added after vortexing in the presence of beads and SUME. The lysate was clarified by centrifugation in an Eppendorf 5415c microfuge at 14,000 rpm for 5 min. The supernatant was transferred to a different tube, and either polyclonal anti-GFP, anti-HA (Covance), or monoclonal anti-FLAG M2 beads (Sigma-Aldrich) were added depending on the substrate. The lysates were nutated overnight at 4°C. In the case of anti-GFP and anti-HA pulldown, 100 μL of protein A Sepharose beads was then added and allowed to nutate for an additional 2 h at 4°C. The beads were then spun down in an Eppendorf 5415c at 1,000 rpm, washed three times with IP wash buffer (50 mM NaCl, 10 mM Tris, pH 7.5), and aspirated to dryness before addition of electrophoretic sample buffer.

Phenotyping. To evaluate cell growth, plate dilution assays were carried out by growing all strains in supplemented minimal medium overnight. A total of 0.35 OD units was centrifuged and resuspended in 1 mL of sterile water. Five- or 10-fold dilutions were then performed

in a 96-well plate and spotted onto on the indicated media. Studies of ethanol sensitivity were conducted using YPD plates with the appropriate ethanol concentration. EtOH plates as well as the YPD control plates were then wrapped in parafilm to prevent ethanol evaporation and grown for 3–7 days at various temperatures.

Confocal Microscopy. All images were taken with a Leica DMI6000 inverted microscope outfitted with a Yokogawa Nipkon spinning disk confocal head, an Orca endoplasmic reticulum high-resolution black and white cooled CCD camera (6.45 μm/pixel at 1×), a Leica Plan Apochromat 40× 1.25 n.a. and 63× 1.4 n.a. objective, and an argon/krypton 100-mW air-cooled laser for 488/568/647-nm excitations. All images were acquired in the dynamic range of 8 bits. Images were analyzed with ImageJ (US National Institutes of Health, Bethesda, MD).

1. Gardner R, et al. (1998) Sequence determinants for regulated degradation of yeast 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* 9:2611–2626, andcorrection (1999) 10:precedi.
2. Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic Acids Res* 21:3329–3330.
3. Cronin S, Hampton RY (1999) Measuring protein degradation with green fluorescent protein. *Methods Enzymol* 302:58–73.
4. Garza RM, Sato BK, Hampton RY (2009) In vitro analysis of Hrd1p-mediated retrotranslocation of its multispanning membrane substrate 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. *J Biol Chem* 284:14710–14722.

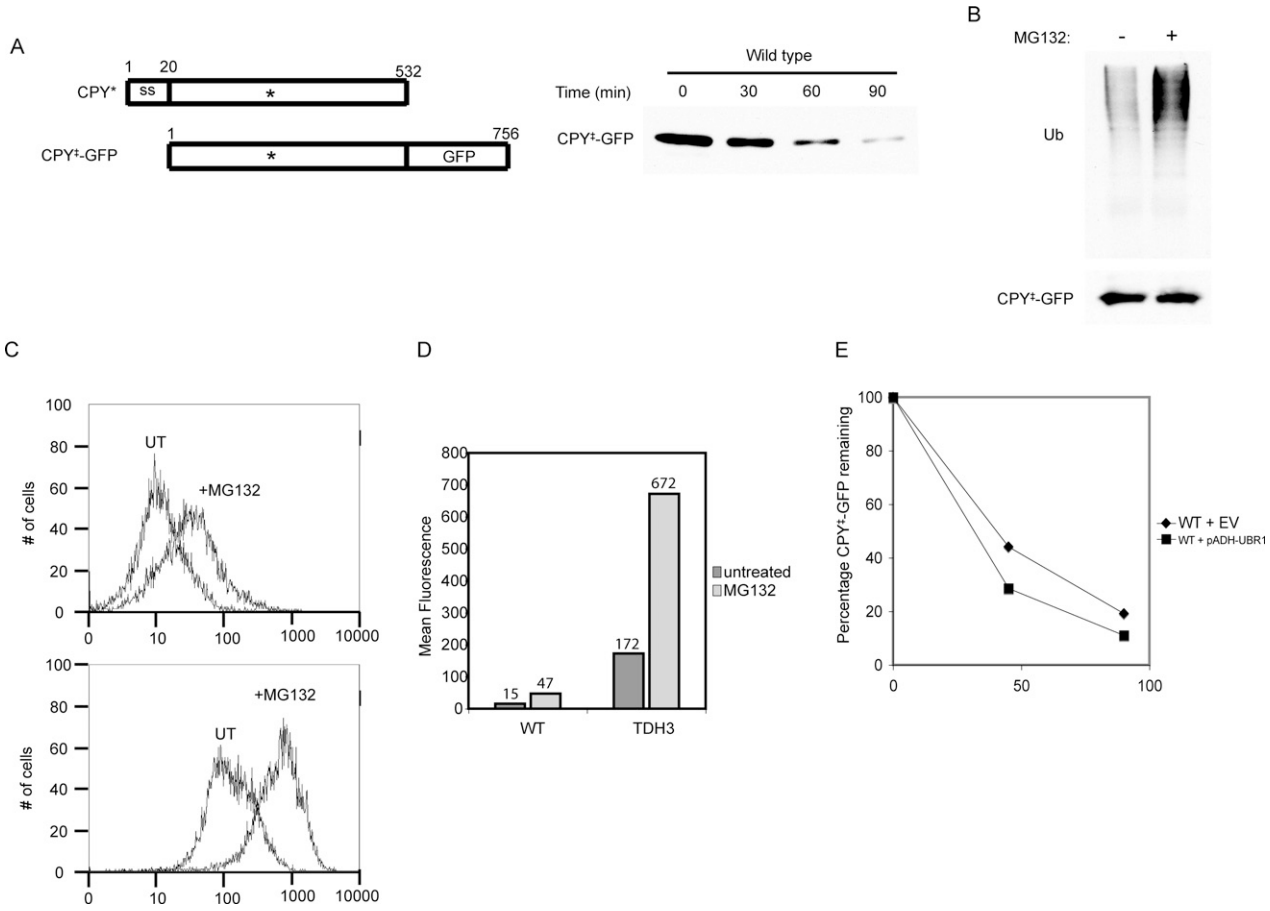

Fig. S1. Characterization of P_{TDH3}-CPY‡-GFP degradation. (*A*) Graphic representation of CPY* and CPY‡-GFP (Δss-CPY*-GFP), in which ss denotes the endoplasmic reticulum localization signal sequence and cycloheximide chase of CPY‡-GFP expressed in WT cells. Anti-GFP antibodies were used to detect CPY‡-GFP. (*B*) Effect of proteasome inhibitor (MG132, 1 h) on in vivo CPY‡-GFP ubiquitination, assayed by anti-GFP IP, followed by antiubiquitin (Ub) or anti-GFP immunoblotting. (*C*) Effect of MG132 on CPY‡-GFP steady-state levels when expressed from native promoter (*Upper*) or strong *TDH3* promoter (*Lower*), as measured by flow cytometry for GFP fluorescence. UT, untreated. (*D*) Mean fluorescence for the histograms in *C* is plotted for each strain, using arbitrary fluorescence units. Magnitudes are written above each bar. (*E*) Overexpression of Ubr1 results in increased degradation rate of CPY‡-GFP. WT cells with empty vector plasmid (EV) or highly expressing ADH promoter-driven *UBR1* plasmid.

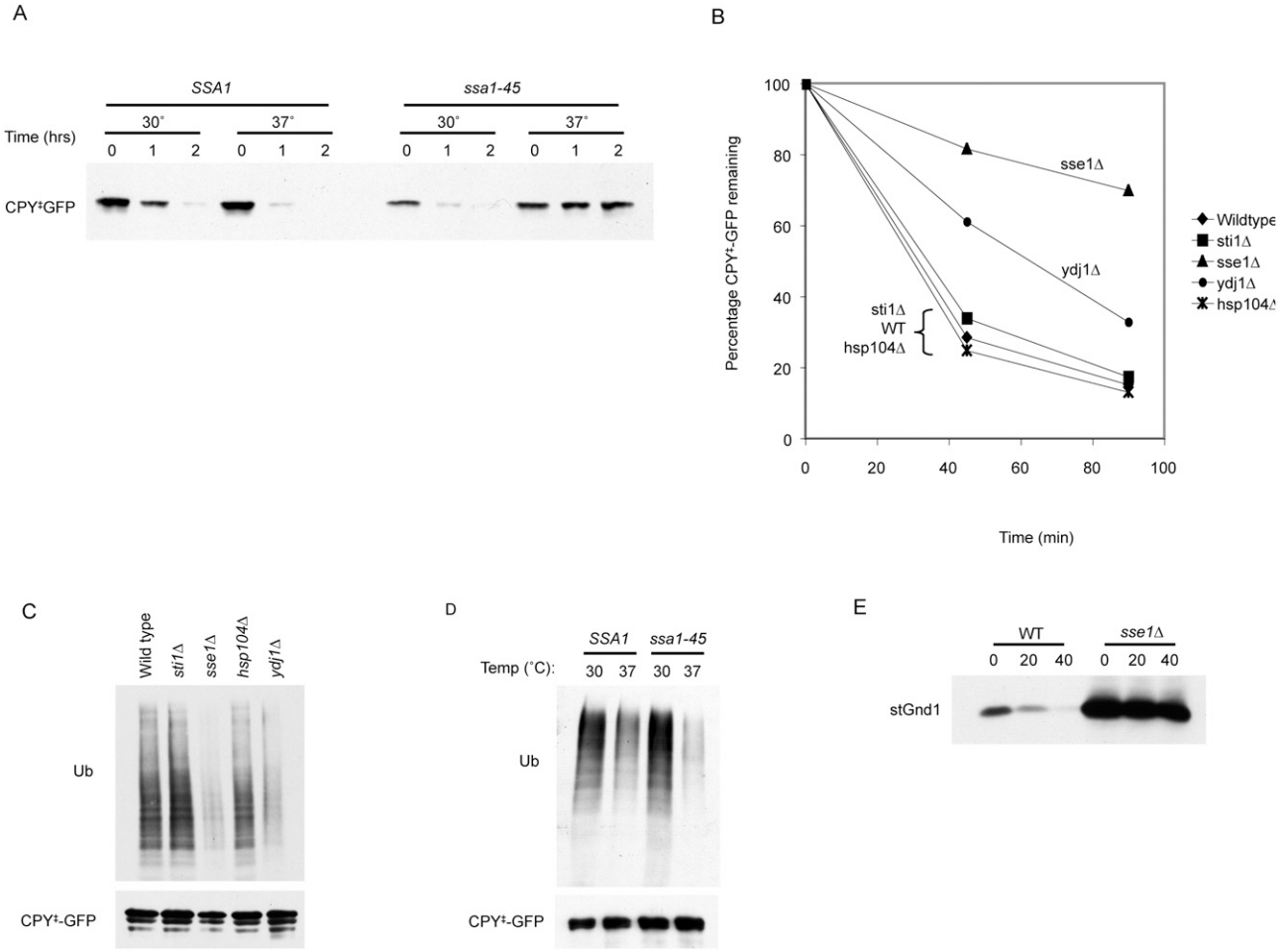

Fig. S2. Chaperone-dependent degradation of QC substrates. (*A*) CPY‡-GFP degradation in *ssa2Δssa3Δssa4Δ* nulls with either WT *SSA1* or temperature-sensitive *ssa1–45* present, evaluated by cycloheximide chase at 30°C or 37°C, followed by anti-GFP immunoblotting. (*B*) Flow cytometry analysis of cycloheximide chase of CPY‡-GFP in chaperone nulls in WT, *sti1Δ*, *sse1Δ*, *ydj1Δ*, and *hsp104Δ*. Mean fluorescence of CPY‡-GFP at each time point was normalized to the steady state at time 0 and graphed as percentage remaining. (*C*) In vivo ubiquitination of CPY‡-GFP expressed in chaperone nulls used in *B*, IP with anti-GFP, and immunoblot with antiubiquitin (Ub) or anti-GFP. (*D*) Ubiquitination of CPY‡-GFP in WT or *ssa1–45* strains used in *A*, at 30°C or 37°C. Cells were pre-incubated at the indicated temperature for 1 h before lysis and IP. Western blots were probed with anti-Ub or anti-GFP. (*E*) Cycloheximide chase of stGnd1 in WT and *sse1Δ* null.

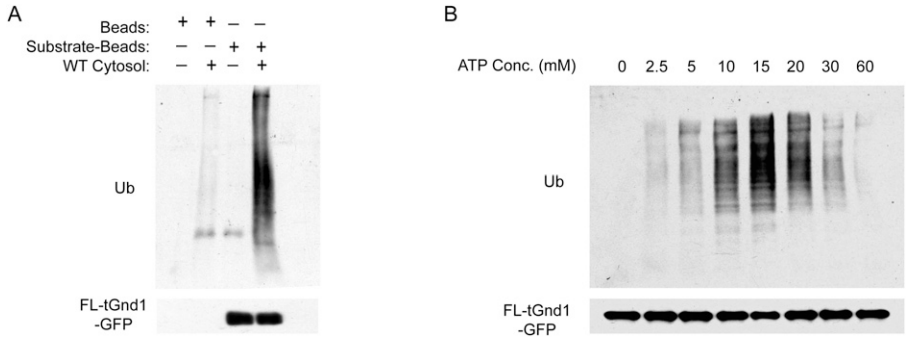

Fig. S3. In vitro ubiquitination assay characterization. (*A*) In vitro ubiquitination of FLAG-tGnd1-GFP. Anti-FLAG agarose beads with bound FLAG-tGnd1-GFP (Substrate-Beads) or untreated anti-FLAG agarose beads (Beads) were incubated with WT cytosol (+) or buffer (–) for 1 h at 30°C. The beads were then washed and resuspended in sample buffer before SDS/PAGE and immunoblotting for ubiquitin (Ub) or GFP. (*B*) ATP-dependent in vitro ubiquitination. Indicated ATP concentrations were added to 150 mg of total cytosol protein and 10 μL of bead-bound tGnd1-GFP, incubated at 30°C for 1 h, and immunoblotted for Ub or GFP.

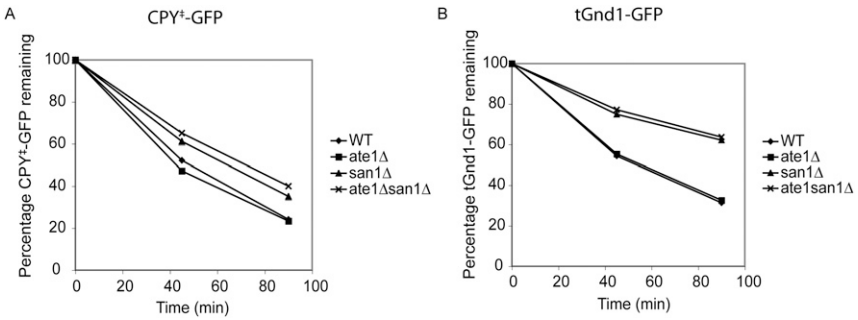

Fig. S4. No effect of ate1Δ on CPY‡-GFP or tGnd1-GFP degradation. (*A*) Flow cytometry analysis of CPY‡-GFP degradation in WT, ate1Δ, san1Δ, and ate1Δsan1Δ nulls. Mean fluorescence of CPY‡-GFP at each time point was normalized to the steady state at time 0 and graphed as percentage remaining. (*B*) Flow cytometry analysis of tGnd1-GFP degradation in strains used in *A*.

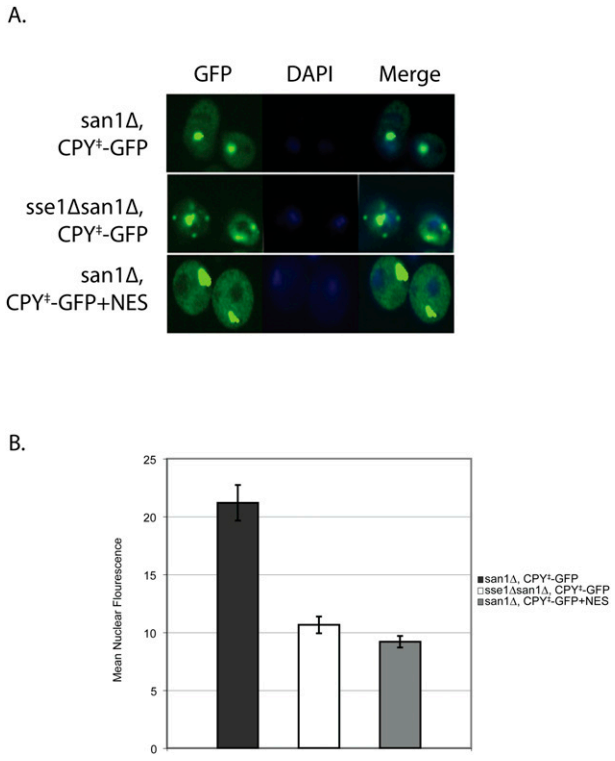

Fig. S5. Sse1-mediated nuclear localization of CPY‡-GFP. (*A*) Representative images of fluorescence microscopy carried out on san1Δ and sse1Δsan1Δ cells expressing CPY‡-GFP as well as san1Δ cells expressing CPY‡-GFP+NES. GFP and DAPI staining was captured to demonstrate the change in localization of CPY‡-GFP within the cell in the absence of sse1Δ. CPY‡-GFP accumulates in the nucleus of san1Δ cells but is restricted to the cytoplasm in the sse1Δsan1Δ cells. (*B*) Quantitation of the mean nuclear signal intensity of GFP fluorescence in the nucleus in san1Δ and sse1Δsan1Δ using ImageJ software, computed as the ratio of DAPI colocalized GFP signal divided by the total GFP signal in the cell. CPY‡-GFP+NES localization in san1Δ cells was used to gauge the lower limits of detection. Error bars are SEM (n = 25 in each condition).

Table S1. *Saccharomyces cerevisiae* strains used in these studies, with RHY designation, relevant markers, plasmids, and origin of strains*

Name	Genotype	Source
BY4741	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Resgen Deletion Collection
RHY4622	JN516; *MATα ura3–52 leu2–3 his3–11, 15 trp1Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Jeff Brodsky
RHY4623	JB67; *MATα ura3–52 leu2–3 his3–11, 15 trp1Δ1 lys2 ssa1::ssa1–45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Jeff Brodsky
RHY6336	BY4741 pRH2081 (P_{TDH3}-*CPY**-*GFP, URA3*)	This study
RHY6337	BY4741 *sti1Δ::KanMX* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY6338	BY4741 *sse1Δ::KanMX* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY6364	*MATα ade2–101 met2 lys2–801 ura3–52 trp1::hisG leu2Δ his2Δ200 CDC34::cdc34-2* pRH2047 (P_{TDH3}-*CPY**-*GFP, URA3*)	
RHY7135	BY4741 ubr1Δ::KanMX pRH2081 (P_{TDH3}-CPY*-GFP, *ADE2 URA3*)	This study
RHY7136	BY4741 *san1Δ::NatMX ubr1Δ::KanMX* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY7157	BY4741 san1Δ::NatMX pRH2081 (P_{TDH3}-CPY*-GFP, *ADE2 URA3*)	This study
RHY7161	BY4741 *san1Δ::NatMX ubr1Δ::KanMX* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*) pRH2439 [*San1(-NLS)-3HSV*]	This study
RHY7165	RHY7136 pRH2444 (*UBR1, LEU2, YCp*)	This study
RHY7169	RHY7136 pRH2445 (*UBR1MR1, LEU2, YCp*)	This study
RHY7447	BY4741	Resgen Deletion Collection
RHY7448	BY4741 *san1Δ::NatMX*	This study
RHY7449	BY4741 *ubr1Δ::KanMX*	Resgen Deletion Collection
RHY7450	BY4741 *san1Δ::NatMX ubr1Δ::KanMX*	This study
RHY7616	RHY7447 pRH2460 (*tFAS1–3HA, URA3,* 2μ)	This study
RHY7617	RHY7448 pRH2460 (*tFAS1–3HA, URA3,* 2μ)	This study
RHY7617	RHY7449 pRH2460 (*tFAS1–3HA, URA3,* 2μ)	This study
RHY7618	RHY7450 pRH2460 (*tFAS1–3HA, URA3,* 2μ)	This study
RHY7620	RHY7447 pRH2476 (P_{TDH3}-*3HA-tGnd1-GFP ADE2 URA3*)	This study
RHY7621	RHY7448 pRH2476 (P_{TDH3}-*3HA-tGnd1-GFP ADE2 URA3*)	This study
RHY7622	RHY7449 pRH2476 (P_{TDH3}-*3HA-tGnd1-GFP ADE2 URA3*)	This study
RHY7623	RHY7450 pRH2476 (P_{TDH3}-*3HA-tGnd1-GFP ADE2 URA3*)	This study
RHY7630	BY4741 *pdr5Δ::KanMX* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY7782	RHY7447 pRH2486 (P_{TDH3}-*FLAG-tGnd1-GFP, ADE2 URA3*)	This study
RHY7783	RHY7448 pRH2486 (P_{TDH3}-*FLAG-tGnd1-GFP, ADE2 URA3*)	This study
RHY7784	RHY7449 pRH2486 (P_{TDH3}-*FLAG-tGnd1-GFP, ADE2 URA3*)	This study
RHY7785	RHY7450 pRH2486 (P_{TDH3}-*FLAG-tGnd1-GFP, ADE2 URA3*)	This study
RHY7873	RHY7447 pRH2491 (P_{TDH3}-*3HAtYor296w, ADE2 URA3*)	This study
RHY7874	RHY7448 pRH2491 (P_{TDH3}-*3HAtYor296w, ADE2 URA3*)	This study
RHY7875	RHY7449 pRH2491 (P_{TDH3}-*3HAtYor296w, ADE2 URA3*)	This study
RHY7876	RHY7450 pRH2491 (P_{TDH3}-*3HAtYor296w, ADE2 URA3*)	This study
RHY7878	BY4741 *sse1Δ::KanMX san1Δ::NatMx*	This study
RHY7983	RHY7878 pRH2474 (P_{ADH1}, *LEU2,* 2μ)	This study
RHY7984	RHY7878 pRH2471 (P_{ADH1}-*UBR1, LEU2,* 2μ)	This study
RHY7987	RHY7447 pRH2516 (P_{TDH3}-*3HA-stGnd1 ADE2 URA3*)	This study
RHY7988	RHY7448 pRH2516 (P_{TDH3}-*3HA-stGnd1 ADE2 URA3*)	This study
RHY7989	RHY7449 pRH2516 (P_{TDH3}-*3HA-stGnd1 ADE2 URA3*)	This study
RHY7990	RHY7450 pRH2516 (P_{TDH3}-*3HA-stGnd1 ADE2 URA3*)	This study
RHY7993	BY4741 *ydj1Δ::Leu2* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY7994	BY4741 *hsp104Δ::Leu2* pRH2081 (P_{TDH3}-*CPY**-*GFP, ADE2 URA3*)	This study
RHY8075	BY4741 *sse1Δ::KanMX*	Resgen Deletion Collection
RHY8198	RHY7447 pRH2531 (P_{TDH3}-*3HA-Gnd1-GFP ADE2 URA3*)	This study
RHY8199	RHY7448 pRH2531 (P_{TDH3}-*3HA-Gnd1-GFP ADE2 URA3*)	This study
RHY8200	RHY7449 pRH2531 (P_{TDH3}-*3HA-Gnd1-GFP ADE2 URA3*)	This study
RHY8201	RHY7450 pRH2531 (P_{TDH3}-*3HA-Gnd1-GFP ADE2 URA3*)	This study
RHY8308	RHY7450 pRH2471 (P_{ADH1}-*UBR1, LEU2,* 2μ)	This study
RHY8309	RHY7450 pRH2439 (*SAN1-NLS, LEU2*)	This study
RHY8368	BY4741 *sse1Δ::KanMX ubr1Δ::LEU2*	This study

*When requesting, please refer to the strain or plasmid number.