Supporting Information

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SI Materials and Methods

Yeast Strains, Media and Growth Conditions. Yeast strains used in this study are listed in Table S1. Most strains are from the *Saccharomyces* Genome Deletion Project (1) which was purchased from Open Biosystems. Double knockouts were generated by PCR-based gene deletion using pFA6a-His3MX6 as a template for homologous recombination (2). Diagnostic PCR using gene-specific primer sets confirmed correct gene deletions in all strains used in this study. Yeast cells were cultured in YPD or synthetic complete (SC) media as previously described (3).

Construction of Expression Plasmids and Transformation. A single copy yeast vector was used for glyceradehyde-3-phosphate dehydrogenase (GPD) gene promoter-mediated constitutive expression (4) unless specified. For the generation of expression plasmids of green fluorescent protein (GFP) or triple hemagglutinin (HA) epitope-fusion proteins, *Not*I restriction enzyme site-flanked GFP or 3 tandem HA epitopes were inserted into a *Not*I site artificially generated at the N or C-terminal end. A plasmid containing four tandem copies of the 22 nucleotide unfolded protein response element (UPRE) inserted upstream of a disabled CYC1 promoter lacZ gene fusion (5) was kindly provided by Peter Walter (University of California, School of Medicine, San Francisco). A Ste6* expression plasmid (6) was generously provided by Susan Michaelis (John Hopkins School of Medicine, Baltimore). Site-directed mutagenesis was conducted by the primer overlap extension method (7). Plasmids were transformed into yeast using the lithium acetate procedure (8). Yeast cells were grown at 30 °C on synthetic complete (SC) media for plasmid selection.

Preparation of Cell Extracts and Immunoblotting. All immunoblotting was performed essentially as described previously (3). Briefly, total protein extracts were prepared by vortexing yeast cells with glass beads in PBS containing protease inhibitor mixture (Complete Mini; Roche) and 1% Triton X-100. Lysates were denatured in SDS sample buffer containing 25 mM DTT for 15 min at 37 °C and resolved by SDS-PAGE before transfer to nitrocellulose membrane and antibody probing.

Immunoprecipitation for Detection of Ubiquitination. Cells were broken by a glass bead vortexing in PBS containing protease inhibitors (Complete Mini; Roche), 5 mM N-ethylmaleimide, 1 mM PMSF, and 1% Triton X-100. HA epitope-tagged proteins were immunoprecipitated with an HA-Tag IP/Co-IP kit (Pierce) according to the manufacturer's specifications. Proteins were eluted by incubation of immobilized anti-HA agarose beads in 2× SDS sample buffer at 37 °C for 15 min and reduced in the presence of 0.1 M DTT for an additional 15 min at 37 °C. Ubiquitin conjugated proteins were detected by immunoblotting using mouse monoclonal antibodies against ubiquitin (Covance).

Microsome Preparation and Chemical Cross-Linking. Microsome preparation was performed as previously described with modifications (9). Cells (A_{600} = 30) were disrupted by glass bead vortexing in 300 μL lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 50 mM potassium acetate (KoAc), 2 mM EDTA, 0.1 M sorbitol, 1 mM DTT, 1 mM phenylmethanesulphonylfluoride (PMSF), and HALT protease inhibitor mixture (Pierce)] for ten 30-sec intervals alternating with incubation on ice. An additional 250 μL of buffer 88 [20 mM Hepes, 50 mM KOAc, 250 mM sorbitol, and 5 mM magnesium acetate (MgOAc)] was added. Unbroken cells and glass beads were cleared by low speed centrifugation (3,000 rpm for 5 min) and then repeated with a second rinse with buffer 88. The supernatant fractions were pooled and centrifuged at 18,000 × *g* for 20 min at 4 °C. The supernatant was decanted and the final microsomal membrane pellet was washed once and re-suspended in 150 μL of 0.2 M triethanolamine, pH 8.0 and incubated on ice for 1 h with 100 μg/mL dimethyl 3,3′-dithiobispropionimidate (DTBP) a water soluble, membrane permeable, and thiol reversible cross-linker. Cross-linking reactions were quenched by addition of 40 μL of 1 M Tris (pH 7.5) and incubated on ice for an additional 20 min. Cell pellets were collected by centrifugation at 18,000 × *g* for 15 min at 4 °C and washed with 400 μL PBS. For membrane solubilization, cell pellets were resuspended in 100 μL of 1% SDS in PBS and incubated at 37 °C for 30 min. Triton X-100 was added for a total concentration of 1.5% (400 μL total volume) and incubated on ice for an additional 30 min. From this lysate, HA-tagged proteins were immunoprecipitated with anti-HA antibody-conjugated Sepharose beads (Pierce) overnight at 4 °C according to the manufacturer. Immobilized proteins were eluted with 2× SDS sample buffer at 37 °C for 15 min. Chemical cross-linking was reversed by incubation with 0.1 M DTT for an additional 15 min at 37 °C before SDS/PAGE and immunoblotting.

β-Galactosidase Reporter Assay. Cells were assayed for β-galactosidase expression as described previously (10). Briefly, cells (A_{600} = 1) were permeabilized with chloroform and 0.1% SDS in 1 mL Z buffer and incubated with 0.2 mL of 4 mg/mL ONPG (*ortho*-Nitrophenyl-β-galactoside) dissolved in Z buffer. Reactions were quenched by adding 0.5 mL Na_2CO_3. β-Galactosidase levels were converted to Miller units $[(A_{420})/(T_{min})(V_{mL})(A_{600})]$.

Oligomycin Resistance Assay. Wild-type and Δ*doa10* strains expressing empty vector, Yor1-GFP or Pca1 (1-392)-Yor1-GFP were cultured in SC selective media to mid-log phase. Cells (≈5 μL, A_{600} = 0.1) were spotted on solid YPEG media (2% Bacto-peptone, 1% yeast extract, 2% ethanol, 3% glycerol, and 1.5% agar), prepared with or without the addition of cadmium (10 μM $CdCl_2$) and oligomycin (2.5 μg/mL). Plates were incubated at 30 °C for 2 days before photography.

Purification and In Vitro Trypsin Proteolysis of Pca1 (250-350). The coding sequence of Pca1 residues 250-350 [Pca1 (250-350)] was cloned into a glutathione sulfur transferase (GST) fusion expression vector, pGEX-6p-1 (Amersham Pharmacia Biotech Inc.) The GST-Pca1 (250-250) was expressed in *Escherichia coli* and cell lysates were prepared. The glutathione Sepharose-immobilized GST-Pca1 (250-250) was digested in metal-binding buffer (100 mM Tris-HCl, pH 7.0, 50 mM NaCl, 100 mM sucrose, 10% glycerol, 1 mM DTT, and 0.1% Triton X-100) with PreScission protease (Amersham) to collect Pca1 (250-350). Pca1 (250-350) peptides were incubated with $CdCl_2$ (0, 50, or 100 μM) at room temperature for 15 min. For limited trypsin proteolysis, the peptides were incubated with trypsin (1 μg/mL final concentration) (Sigma) for 10 min on ice before addition of soybean trypsin inhibitor (0.2 μg/mL final concentration) (Fluka BioChemika) for an additional 15 min on ice. Proteolysis patterns were visualized by Coomassie blue staining.

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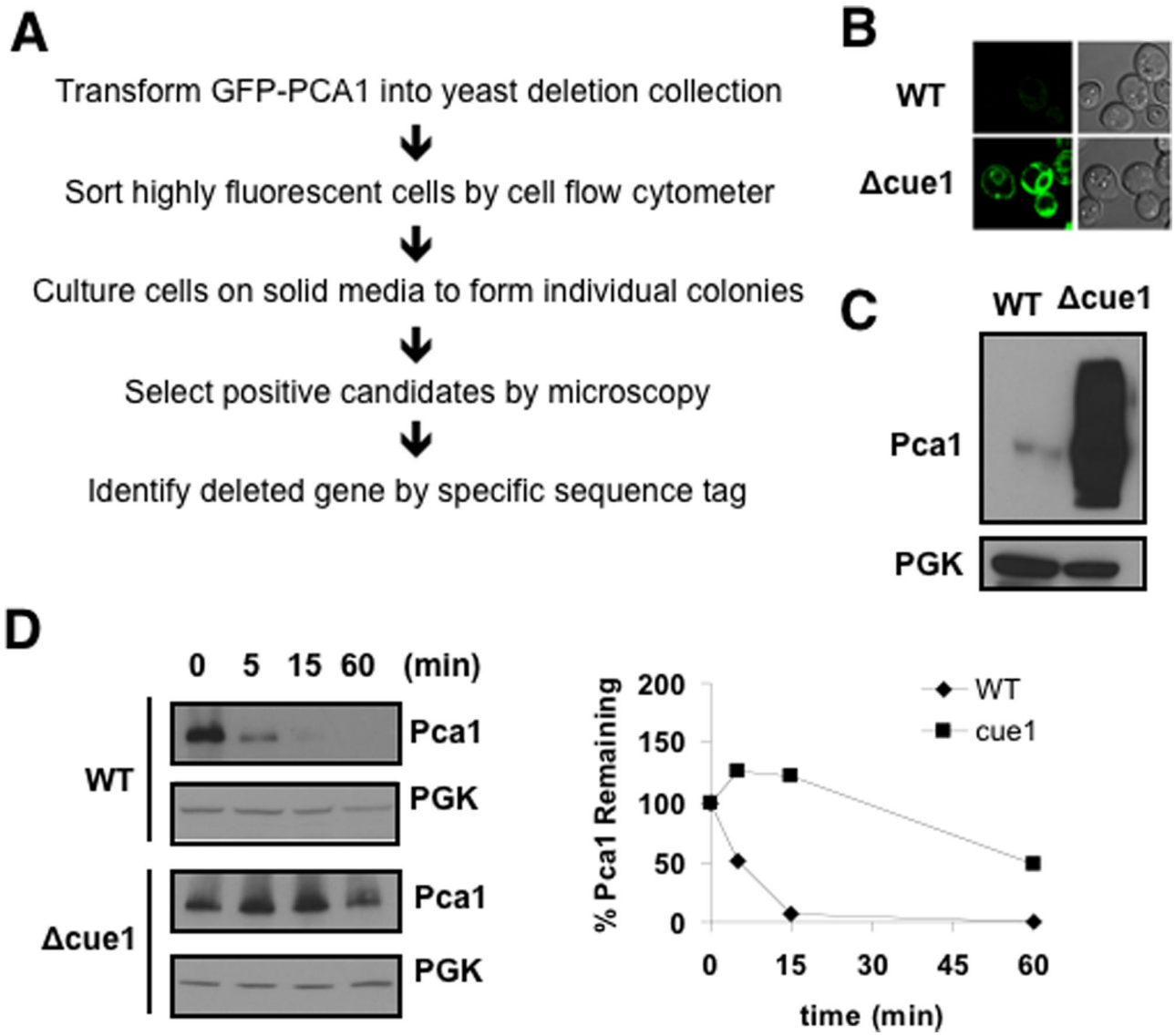

Fig. S1. Genetic screen conducted to identify factors involved in Pca1 turnover. (*A*) Schematic of the strategy for identifying factors involved in Pca1 turnover. (*B*) Confocal fluorescence microscopy of wild-type (WT) or Δ*cue1* cells expressing GFP-fused Pca1. (*C*) Western blot of HA epitope-tagged Pca1 expressed in Δ*cue1* cells. Phosphoglycerate kinase (PGK) was probed as a loading control. (*D*) Cycloheximide chase and western blotting of HA-Pca1 in a Δ*cue1* strain. Each blot was probed for PGK to determine equal loading. Pca1 levels at each time point were measured by the quantification of pixel densities and normalized to those of PGK (*Right*). Data are representative of at least 3 independent experiments.

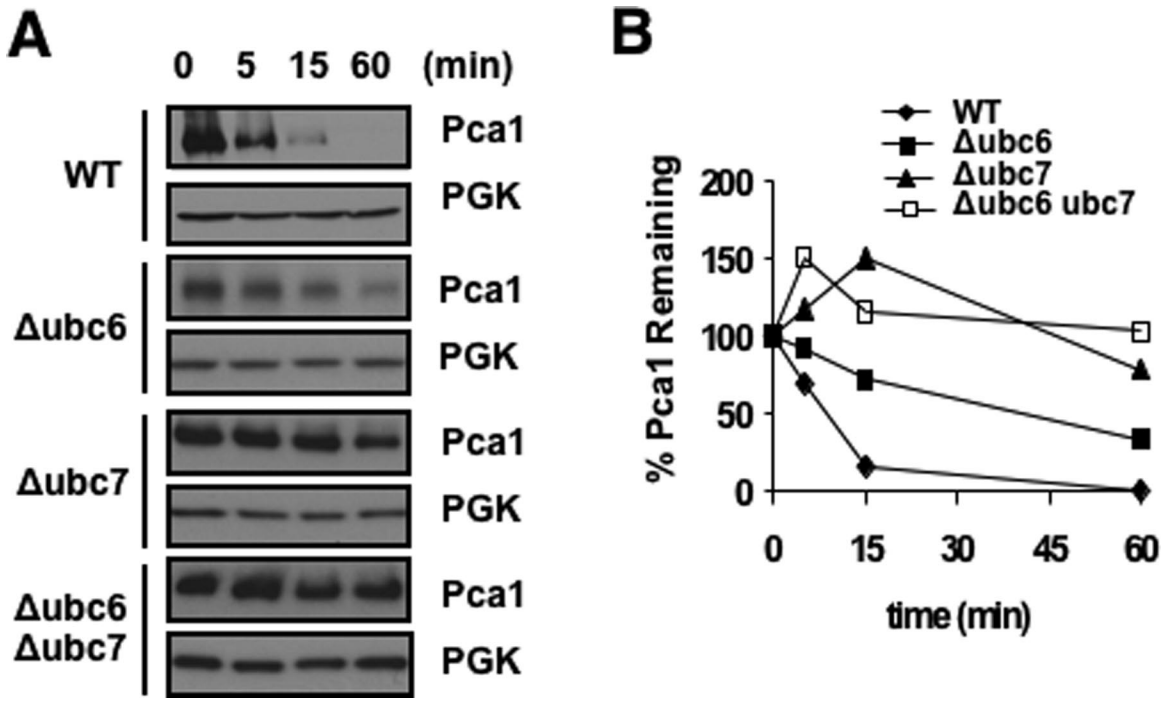

Fig. S2. Roles for Ubc7 and Ubc6 ubiquitin-conjugating enzymes in Pca1 turnover. (*A*) Cycloheximide chase and immunoblotting of HA epitope-tagged Pca1 expressed in WT, Δ*ubc6*, Δ*ubc7*, or Δ*ubc6* Δ*ubc7* strains at the indicated time points. Representative data from 3 independent experiments is shown. (*B*) For quantification, pixel densities of Pca1 were normalized to those of PGK.

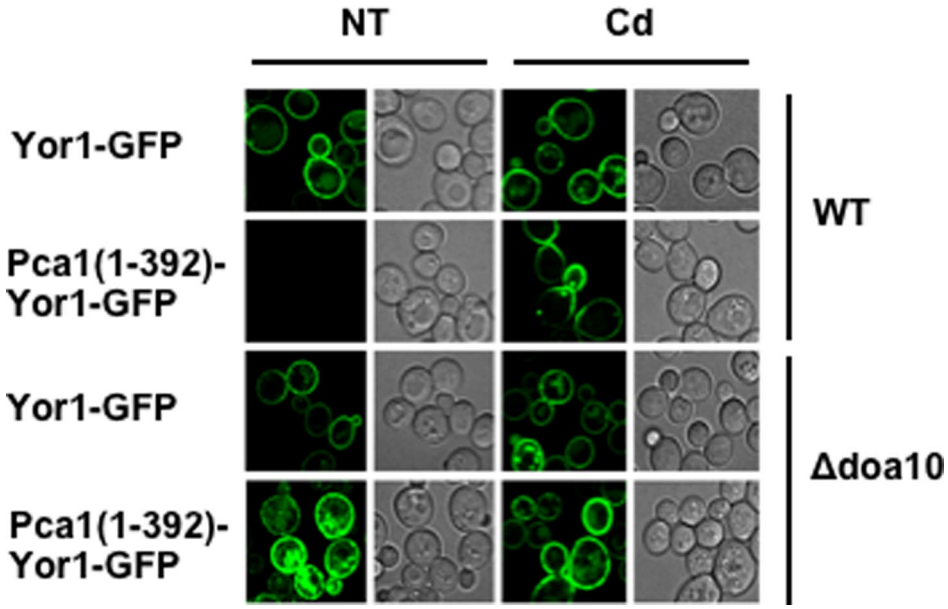

Fig. S3. Doa10 and cadmium-dependent turnover of Yor1 fused with the Pca1 N-terminal regulatory domain. Empty vector, Yor1-GFP, or Pca1 (1-392)-Yor1-GFP expression constructs were transformed in WT and Δ*doa10* strains. Cells were cultured in non-treated (NT) liquid media or with cadmium (50 μM $CdCl_2$, 1 h). Relative expression of Yor1-GFP fusion constructs was determined by confocal fluorescence microscopy.

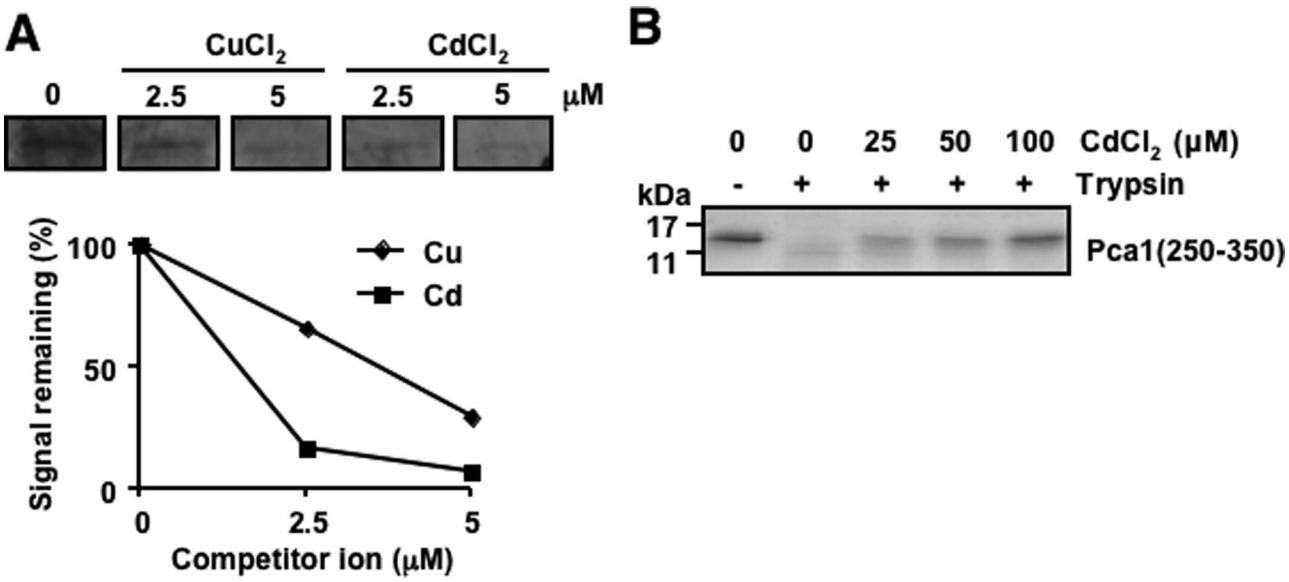

Fig. S4. Metal binding and conformational changes within the Pca1 degron. (*A*) Competition of ^{64}Cu binding by copper and cadmium. Pca1 amino acid residues 250-350 tagged with triple HA epitope at the N terminus [HA-Pca1 (250-350)] were expressed in a Δ*doa10* strain. Immunoprecipitated HA-Pca1 (250-350) were subjected to ^{64}Cu (1 μM $CuCl_2$, 10 μCi) blotting assays followed by autoradiography (see *Material and Methods*). ^{64}Cu blotting was performed in the presence of (0, 2.5, or 5 μM) of copper ($CuCl_2$) or cadmium ($CdCl_2$) competitor ions. Graph (*Lower*) is the quantification of pixel densities. (*B*) Cadmium-dependent protection of Pca1 (250-350) from trypsin proteolysis. Purified Pca1 (250-350) was incubated in metal binding buffer (see *SI Materials and Methods*) with the indicated concentrations of $CdCl_2$ and subjected to limited trypsin proteolysis. Pca1 (250-350) was visualized by Coomassie stain following SDS/PAGE.

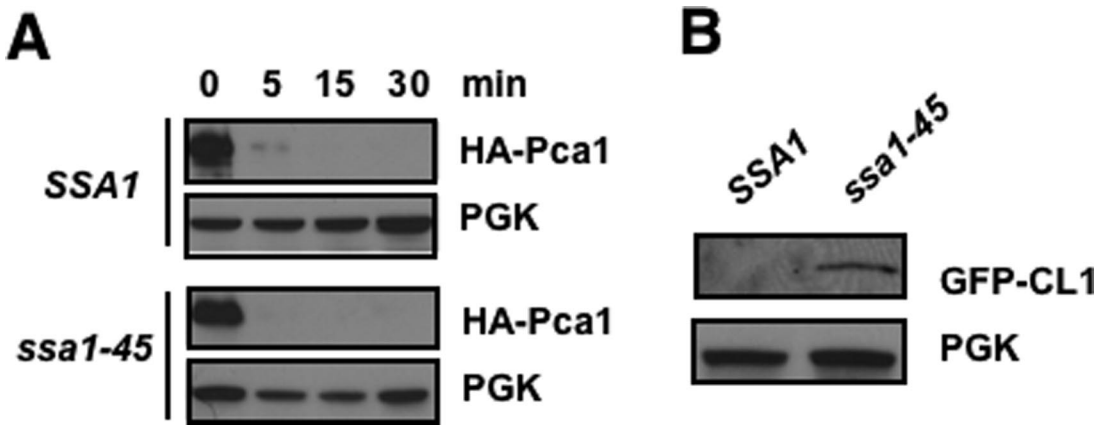

Fig. S5. Hsp70 chaperones do not affect Pca1 turnover. Cycloheximide chase and western blotting of HA-Pca1 (*A*) and GFP-CL1 (*B*) in the *SSA1* and *ssa1–45* strains. In these yeast cells, other cytosolic Hsp70 chaperones, including SSA2, SSA3, and SSA4, were deleted (14). Cells were precultured at 37 °C for 1 h to inactivate ssa1–45 before supplementation of cyclohexamide (100 μg/mL). Expression levels of HA-Pca1 at 0, 5, 15, and 30 min were determined by immunoblotting with anti-HA antibodies. GFP-CL1 expression was measured after culturing cells at 37 °C for 1 h by immunoblotting using anti-GFP antibodies. Each blot was probed for PGK to determine equal loading.

Table S1. Yeast *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Ref.
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4741 Δ*cue1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cue1::KanMX*	Open Biosystems
BY4741 Δ*doa10*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 doa10::KanMX*	Open Biosystems
BY4741 Δ*hrd1*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1::KanMX*	Open Biosystems
BY4741Δ*hrd1* Δ*doa10*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1::KanMX doa10::HIS3*	This study
BY4741 Δ*ubc6*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc6::HIS3*	(11)
BY4741 Δ*ubc7*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc7::KanMX*	Open Biosystems
BY4741 Δ*ubc6* Δ*ubc7*	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubc6::HIS3 ubc7::NatMX*	(11)
SEC23	*MATα ura3–52 leu2–3,-112*	(12)
sec23–1	*MATα ura3–52 his4–619 sec23–1*	(12)
CDC48	*MATa ura3–52 his3Δ200 leu2Δ1 trpΔ63*	(13)
cdc48–1	*MATa ura3–52 his3Δ200 leu2Δ1 trpΔ63 cdc48–3*	(13)
SSA1	*MATα his3–11,3–15leu2–3,2–112 ura3–52 trp1-Δ1 lys2 SSA1 ssa2–1 ssa3–1 ssa4–2*	(14)
ssa1–45	*MATα his3–11,3–15leu2–3,2–112 ura3–52 trp1-Δ1 lys2 ssa1–45BKD ssa2–1 ssa3–1 ssa4–2*	(14)
W303–1b	*MATα ade2 his3 leu2 ura3 trp1 can1–100*	(9)
KNY111	*MATα ade2 his3 leu2 ura3 trp1 can1–100 DOA10–13MYC-KANMX*	(9)