Supporting Information

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SI Materials and Methods

Yeast Strains and Plasmids. Media preparation, genetic, and molecular biology techniques were carried out using standard methods. Yeast strains used in this study contain the genetic background W303 *ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1ocre can1-100*. The strains YWO 343 and YWO 636, which contain a mutated and deleted allele of *PRC1*, respectively, are referred to as wild type. Deletion strains were generated by homolog recombination (1). All strains and plasmids and antibodies used in this study are summarized in Tables S1 and S2, respectively.

Antibodies. CPY antibodies (rabbit; Rockland; 200–401-135) were used to precipitate mutated carboxypeptidase Y (CPY*) and CT* (consisting of a CPY* moiety fused to the last transmembrane domain of Pdr5) in pulse chase (PC) experiments. Ste6* (mutated Ste6; sterile) HA was precipitated with the help of HA 16B12 antibodies (mouse; Covance; MMS-101P). Cdc48 (cell devision cycle) antibodies were provided by Thomas Sommer (Max Delbrück Center for Molecular Medicine, Berlin, Germany). Hydrophilic 8-amino acid peptide with the sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG)-Ubr1, ubiquitin, phosphoglycerol kinase (PGK), CFTR HA, and CPY* were detected on immunoblots with FLAG (mouse; Sigma; F3165), Ub P4G7 (mouse; Covance; MMS-258R), PGK (mouse; Sigma-Aldrich), HA 16B12 (mouse; Covance; MMS-101P), and CPY (mouse; Molecular Probes; A6428) antibodies, respectively.

Ubiquitination Assay. First, 450 OD of cells in logarithmic growth phase were harvested and washed with cold water. After centrifugation, the cells were resuspended in 20 mM ice-cold sodium azide and incubated on ice for 10 min to stop ATP-dependent metabolic pathways. Subsequently, the cells were washed once with and resuspended in 1 mL and 0.5 mL sorbitol buffer [0.7 M sorbitol/50 mM Tris·HCl, pH 7.5/1 mM PMSF/1 μg/μL Pepstatin A/1× complete inhibitor mix (Roche)/20 mM *N*-ethylmaleimide], respectively. After addition of 0.3 mL glass beads (0.4–0.6 mm), the cells were lysed by 10 min of vortexing at 8 °C. Next, 1 mL sorbitol buffer was added, and samples were mixed and centrifuged (10 min; 500 × *g*; 4 °C) to remove large cell fragments and unbroken cells. The supernatant was subjected to ultracentrifugation (35 min; 100,000 × *g*; 4 °C) to separate cytosolic from membrane fractions. Then, 1 mL supernatant of the ultracentrifugation step, containing the cytosolic protein pool, was loaded with 100 μL solubilization buffer [50 mM Tris·HCl, pH 7.5/1% SDS/1 mM PMSF/1× complete inhibitor mix (Roche)] and incubated for 2 h on a rotating disk. The pellet containing the membrane fractions was resuspended in 1 mL immunoprecipitation (IP) buffer [50 mM Tris·HCl, pH 7.5/165 mM NaCl/1.1% Triton X-100/5.5 mM EDTA/1 mM PMSF/1× complete inhibitor mix (Roche)], stowed with 100 μL solubilization buffer, and incubated for 1.5 h on a rotating disk. Nonsolubilized components of the membrane fraction were removed by ultracentrifugation (35 min; 100,000 × *g*; 4 °C). Indicated antibodies were added to the cytosolic protein fraction as well as to the solubilized membrane proteins, and samples were incubated for 1 h. Next, 80 μL of 6.25% protein A Sepharose was added, and the samples were incubated for another 1–2 h on a rotating disk. Precipitates were washed three times with IP buffer, and the precipitated proteins were removed from the Sepharose by addition of 50 μL urea buffer [200 mM Tris·HCl, pH 6.8/8 M urea/0.1 M EDTA/5% SDS (wt/vol)/0.05% bromophenol blue/1% 2-mercaptoethanol] and subjected to SDS/PAGE with subsequent immunoblot.

PC Analysis. PC analysis of CPY* was described previously (2) and carried out for CT* accordingly.

PC analysis for the substrate Ste6* HA and CFTR HA was carried out as follows: Cells were grown in complete minimal medium at 30 °C. Then, 60 OD of cells was harvested in early logarithmic phase, washed three times with starvation medium (3), and resuspended in 1 mL starvation medium. After 50 min starvation, 200 μCi of ^{35}S-labeled methionine was added to label cells. After 30 min of pulse, 2.6 mL of prewarmed chase medium (starvation medium plus 2 mg/mL BSA and 6 mg/mL methionine) was added, and the culture was incubated for an additional 30 min. Cell samples were taken at indicated time points, and metabolism was stopped by incubation with 20 mM NaN_3. After centrifugation and removal of the supernatant, the cells were lysed by vortexing (10 min) with glass beads (0.4–0.6 mm) and 100 μL extraction buffer [50 mM Tris·HCl, pH 7.5/0.5 mM EDTA, pH 8.0/1 mM PMSF/1× complete inhibitor mixture (Roche)]. After the addition of 1 mL extraction buffer, 950 μL of the suspension was transferred to a new test tube, and membranes were isolated from the cytosolic fraction by centrifugation at 16,000 × *g* for 15 min. Membrane proteins were dissolved by incubation with 100 μL solubilization buffer (30 min; 50 mM Tris·HCl, pH 7.5/1% SDS) and then 1 mL IP buffer [30 min; 165 mM NaCl/5.5 mM EDTA, pH 8.0/1.25% Triton X-100/190 mM NaCl/1 mM PMSF/1× complete inhibitor mixture (Roche)]. Nonsolubilized proteins were removed by centrifugation at 16,000 × *g* for 15 min. Immunoprecipitation with 3.5–4 μL HA antibodies, detection, and quantification were performed as described previously (3).

In the case of temperature-sensitive strains, cells were grown at 25 °C and shifted to 37 °C 1 h before the first sample was taken. In cases of proteasomal inhibition, MG132 dissolved in DMSO (end concentration, 150 μM) or an equivalent amount of pure DMSO was added at −30 min and every 30 min thereafter. The heat shock response was induced by shifting cells to 37 °C and 42 °C at −30 min and −60 min, respectively. In cases of ethanol stress, ethanol was added to the starvation and chase media, with a final concentration of 10% (vol/vol). For stress stimulation with tunicamycin, indicated amounts of tunicamycin were added to the media at −60 min. Under this stress condition, the incubation time after addition of chase media was reduced to 3 min.

Cycloheximide Chase Analysis. First, 45 OD of cells in logarithmic phase was harvested and resuspended in 2.6 mL complete minimal selection medium. After addition of 150 μL cycloheximide (10 mg/mL; time point 0), 600 μL cell suspension was taken at indicated time points. ATP-dependent processes were stopped by adding 500 μL NaN_3 (20 mM). After centrifugation and removal of the supernatant, the cells were resuspended in 300 μL potassium phosphate buffer (50 mM, pH 7.5) and 100 μL trichloroacetic acid [55% (wt/vol)]. The samples were incubated for 30 min at −80 °C and subsequently centrifuged for 5 min at 16,000 × *g*. The resulting pellet was washed once with ice-cold acetone [80% (vol/vol)], dried, and resuspended in 80 μL 1% SDS, 0.1 M NaOH. After addition of 20 μL 5× sample buffer [1 M Tris·HCl, pH 6.8/1% (wt/vol) SDS/25% (wt/vol) 2-mercaptoethanol/50% (vol/vol) glycerol/0.05% bromophenol blue], the samples were incubated for 5 min at 45 °C and subjected to immunoblot analyses.

Propidium Iodide Staining. Cells of 0.5–1 mL cell culture were harvested at indicated time points, and were washed once with and

subsequently resuspended in 1 mL CellWash solution (BD). The cell solution was stowed with 10 μL propidium iodide solution (10 mg/mL), incubated for 1 min at room temperature, and subjected to FACS analysis (BD FACSCalibur and provided software).

1. Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* 30(6):e2.
2. Benitez EM, Stolz A, Wolf DH (2011) Yos9, a control protein for misfolded glycosylated and non-glycosylated proteins in ERAD. *FEBS Lett* 585(19):3015–3019.
3. Kohlmann S, Schäfer A, Wolf DH (2008) Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. *J Biol Chem* 283(24):16374–16383.

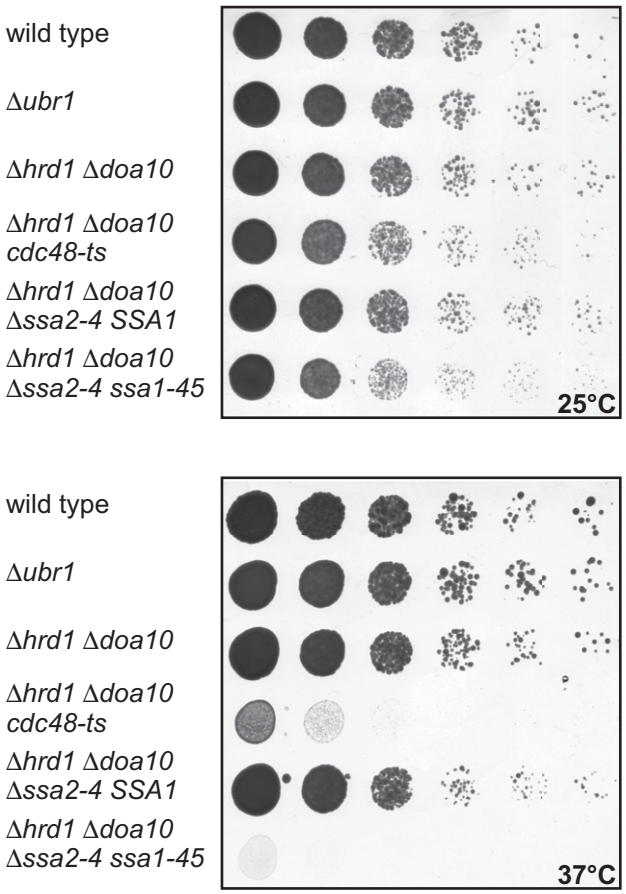

Fig. S1. Strains harboring temperature-sensitive alleles of CDC48 and SSA1 grow comparably to the wild type under permissive temperature. From cells grown to late logarithmic phase, 4.5 OD was harvested and resuspended in 1 mL rich complete media containing yeast extract, peptone and glucose (YPD). One drop of a subsequently generated dilution series (1:10) was transferred onto YPD plates and incubated for 2 d at 25 °C and 37 °C, respectively.

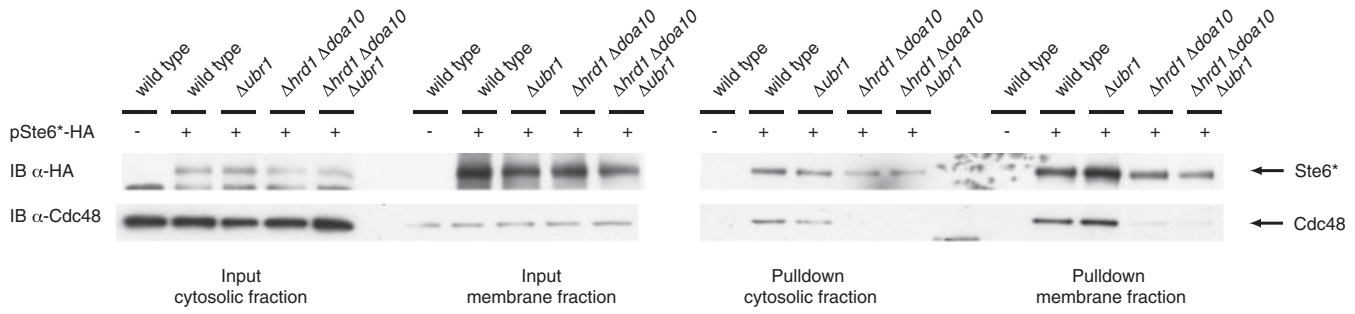

Fig. S2. Cdc48 interacts with the endoplasmic reticulum-associated degradation (ERAD) substrate Ste6*. Cell lysates of indicated strains were separated via ultracentrifugation (100,000 × *g*) into cytosolic and membrane fractions and then subjected to immunoprecipitation under conditions preserving interaction with noncovalent binding partners. Ste6* HA was precipitated with the help of HA antibodies. In subsequent immunoblot analyses, HA antibodies and Cdc48 antibodies were used to detect Ste6* and Cdc48, respectively.

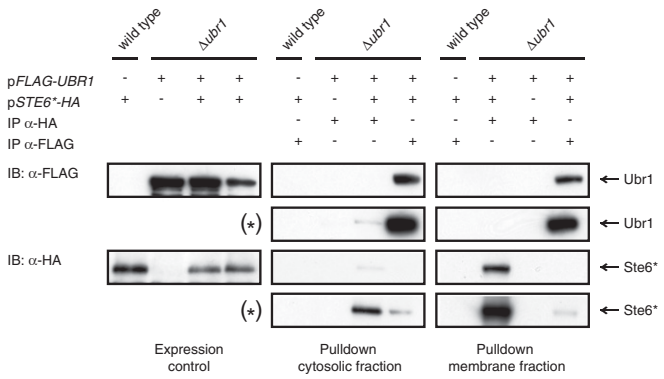

Fig. S3. Ubr1 is in complex with the ERAD substrate Ste6* in wild-type cells. Cell lysates of indicated strains were separated via ultracentrifugation (100,000 × *g*) into cytosolic and membrane fractions and then subjected to immunoprecipitation under conditions preserving interaction with noncovalent binding partners. Only UBR1-deleted cells were complemented with plasmid-encoded FLAG-tagged Ubr1. Plasmid-encoded Ste6* HA and Ubr1-FLAG were precipitated from cytosolic or membrane fraction with the help of HA and FLAG antibodies, respectively. Cells lacking the plasmid-encoding Ste6* HA or expressing untagged wild-type Ubr1 served as negative controls.

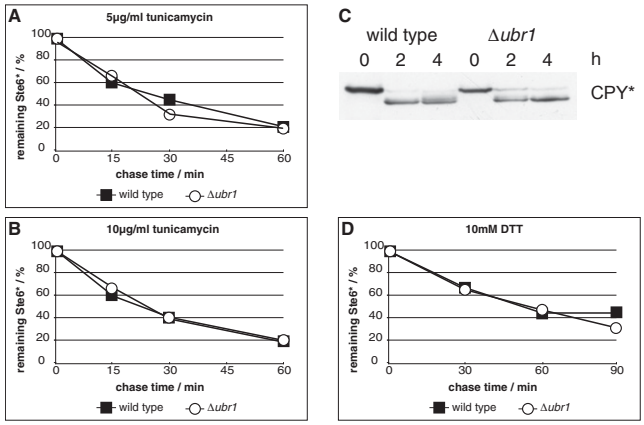

Fig. S4. Exposure to the stress stimuli tunicamycin and DTT does not trigger Ubr1-dependent ERAD of Ste6*. PC (*A* and *B*) and cycloheximide chase analysis (*D*) of Ste6* in the presence of the stress stimuli tunicamycin and DTT, respectively. (*C*) Glycosylation state of CPY* after treatment with 10 μg/mL tunicamycin. Detection of the immunoblot was carried out with CPY antibodies. In nontreated cells, CPY* is known to carry glycan trees on four glycosylation sites.

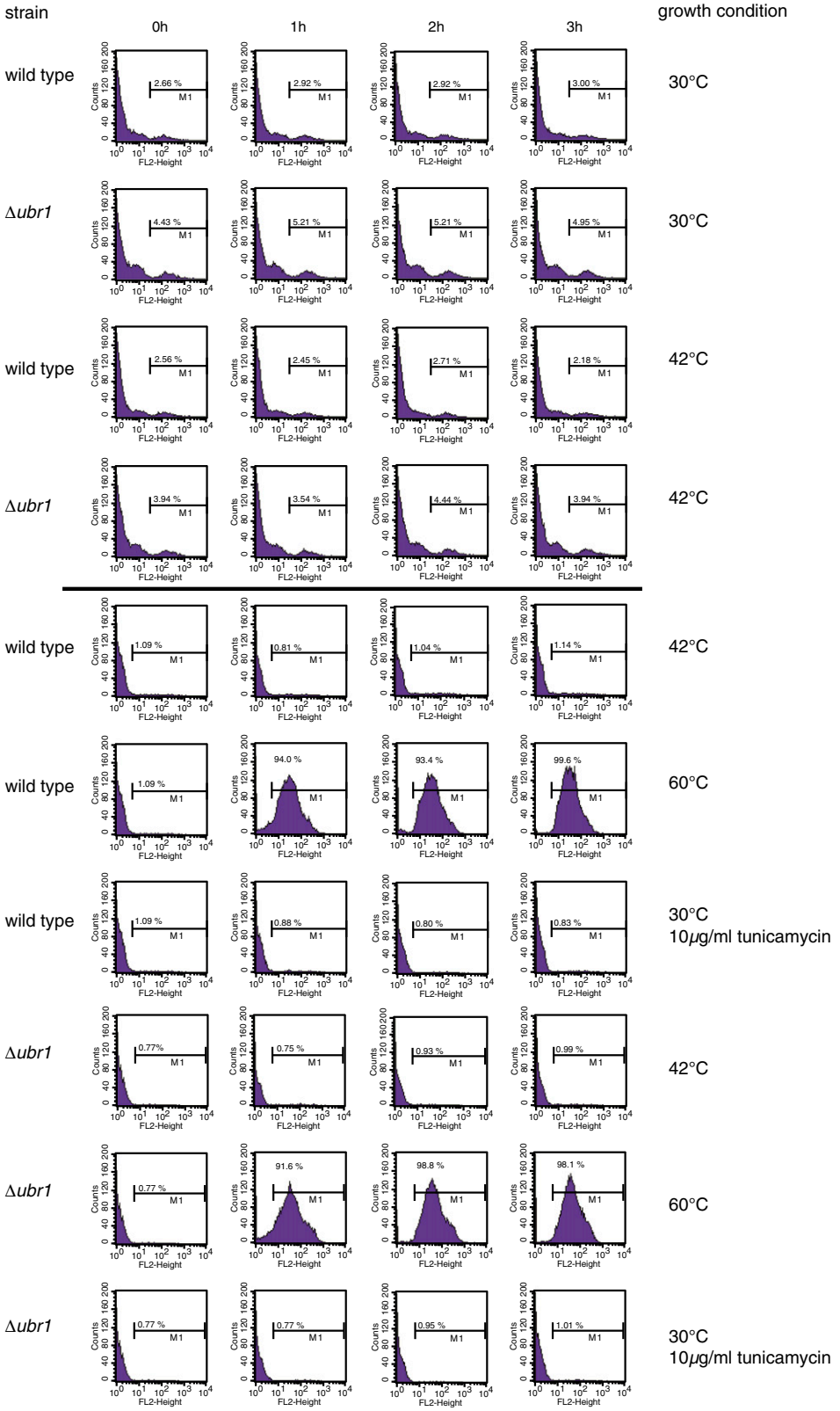

Fig. S5. Cells survive stress conditions of 42 °C or treatment with 10 μg/mL tunicamycin. Cells of indicated strains were harvested in logarithmic phase and according to the PC protocol (*SI Materials and Methods*), starved for 50 min at 30 °C. After this period, chase medium was added (time point 0) and the cells were shifted to the indicated temperatures or treated with tunicamycin. Cell samples were taken at indicated time points, stained with propidium iodide, and analyzed via FACS analysis. Separated panels represent two independent experiments. Massive heat stress (60 °C) leading to cell death was used as a control.

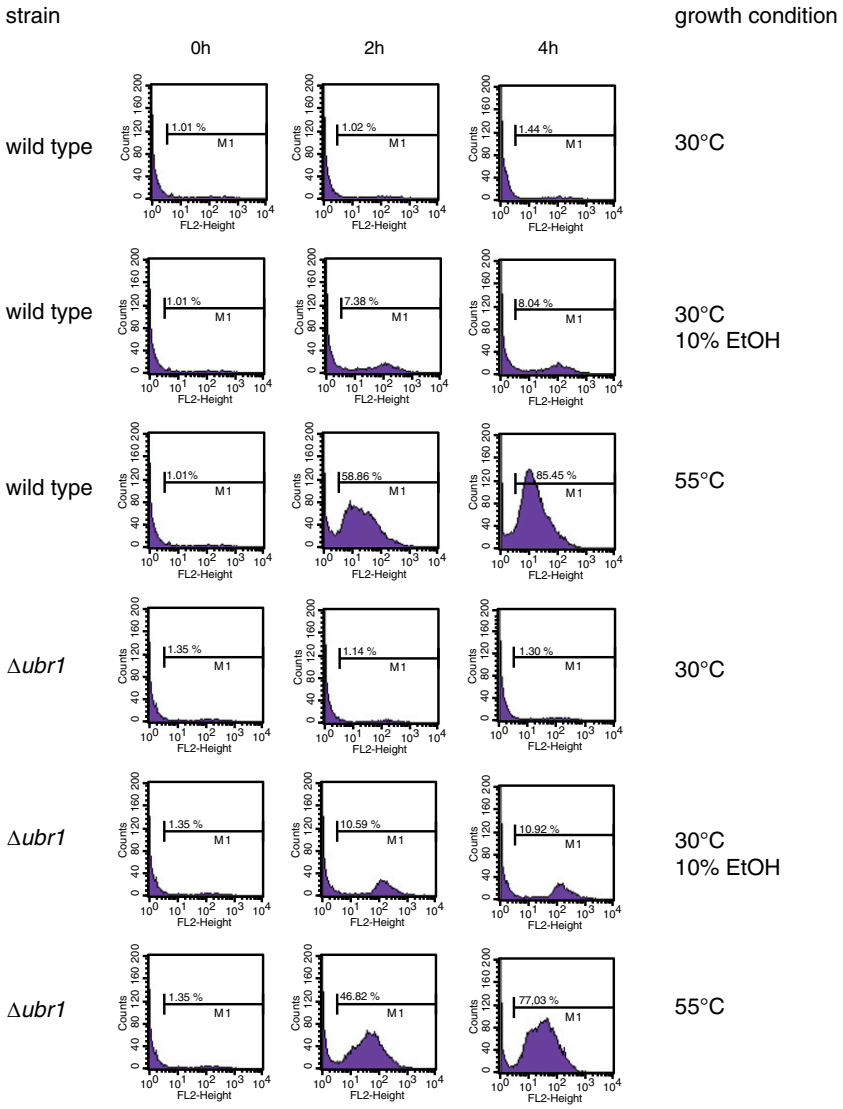

Fig. S6. Cells survive ethanol (10%) stress. Cells of the indicated strains were harvested in logarithmic phase and according to the PC protocol (*SI Materials and Methods*), starved for 50 min at 30 °C. After this period, chase medium was added. Cell samples were taken at indicated time points, stained with propidium iodide, and analyzed via FACS analysis. Ethanol was added to the media (10% final concentration) during starvation and subsequent chase period. Massive heat stress (55 °C) leading to cell death was used as a control.

Table S1. Yeast strains (W303 background) used in this study

Name	Genotype	Source
YWO 343	*prc1-1*	
YWO 636	*prc1Δ::LEU2*	Described in ref. 1
YWO 640	*prc1-1 ubc6Δ::LEU2 ubc7Δ::LEU2 DER3::3HA-HIS5*$^{+}$	P. Deak (Institut für Biochemie, Universität, Stuttgart, Germany)
YWO 1428	*prc1-1 ubr1Δ::HIS5*$^{+}$	Described in ref. 2
YWO 1525/YWO 1527	*prc1-1 hrd1Δ::HIS3 doa10Δ::kanMX*	Described in ref. 3
YWO 1528	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX*	This study
YWO 1648	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX dfm1Δ::HIS3MX6*	This study
YWO 1731	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX ubr1Δ::HIS5*$^{+}$	This study
YWO 1741	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::kanMX pdr5Δ::TRP1*	This study
YWO 1803	*prc1Δ::kanMX hrd1Δ::HIS5*$^{+}$ *doa10Δ::LEU2 cdc48*^{T413R} *(ts)*	This study
YWO 1817	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::loxP ubx6Δ::loxP ubx7Δ::kanMX*	This study
YWO 1820	*prc1Δ::LEU2 hrd1Δ::HIS3 doa10Δ::loxP ubx6Δ::loxP*	This study
YWO 1944	*prc1-1 hrd1Δ::HIS3 dao10Δ::loxP ubr1Δ::loxP*	This study
YWO 1956	*prc1Δ::LEU2 hrd1Δ::loxP doa10Δ::NAT ssa2Δ::loxP ssa3Δ1−1126::loxP ssa4Δ::loxP*	This study
YWO 1957	*prc1Δ::LEU2 hrd1Δ::loxP doa10Δ::NAT ssa1-45 (ts) ssa2Δ::loxP ssa3Δ1−1126::loxP ssa4Δ::loxP*	This study
YWO 1980	*prc1-1 hrd1Δ::HIS3 doa10Δ::kanMX ubc2::loxP*	This study
YWO 2011	*hrd11−prc1Δ::HIS3 doa10Δ::kanMX ubx1Δ::HIS3 ubx7Δ::LEU2*	This study

1. Plemper RK, Deak PM, Otto RT, Wolf DH (1999) Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. *FEBS Lett* 443(3):241–245.
2. Eisele F, Wolf DH (2008) Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Lett* 582(30):4143–4146.
3. Stolz A, Schweizer RS, Schäfer A, Wolf DH (2010) Dfm1 forms distinct complexes with Cdc48 and the ER ubiquitin ligases and is required for ERAD. *Traffic* 11(10):1363–1369.

Table S2. Plasmids used in this study

Name	Insert	Marker	Refs. for description
pCT67/PWO 0804	*CT**	*URA3*	(1)
pSM1911/PWO 1026	*ste6-166::HA*	*URA3*	(2)
pNTFLAG-UBR1	*FLAG-UBR1*	*LEU*	(3)
pSM1152	*CFTR-HA*	*URA*	(4)
pNTFLAG-UBR1-MR1	*FLAG-UBR1-RM*	*LEU*	(3)

1. Taxis C, et al. (2003) Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J Biol Chem* 278(38):35903–35913.
2. Loayza D, Tam A, Schmidt WK, Michaelis S (1998) Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of an ER quality control pathway in Saccharomyces cerevisiae. *Mol Biol Cell* 9(10):2767–2784.
3. Xie Y, Varshavsky A (1999) The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. *EMBO J* 18(23):6832–6844.
4. Zhang Y, Michaelis S, Brodsky JL (2002) CFTR expression and ER-associated degradation in yeast. *Methods Mol Med* 70:257–265.