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Supplemental Data

Dissecting the ER-Associated Degradation of a Misfolded Polytopic Membrane Protein

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Supplemental Experimental Procedures

Plasmids and Strains

Yeast strains used in this study are listed in Table S1. KNY108 was created by PCR based disruption. In brief, a *doa10*::*kanMX4* cassette was made by PCR using pRS400 (Baker et al., 1998) as a template and primers 5'-TAG CCA AGA GTA CCA CTA ATT GAA TCA AAG AGA CTA GAA GTG TGA AAG TCA GAT TGT ACT GAG AGT GCA C-3' and 5'-TAT ATG TAA ATA TGC TAG CAT TCA TTT TAA ATG TAA GGA AGA AAA CGC CTC TGT GCG GTA TTT CAC ACC G-3. KNY100 was transformed with the resulting fragment to give KNY108. Correct integration was confirmed by PCR using primers 5'-TAG CCT TGT TGG CGC AAT CGG TA -3' and 5'-GAA CTG AAC GAA AAG TTG GTT GA-3', which localize in the 5'-UTR and 3'-UTR, respectively. KNY109, KNY110, and KNY111 were created by PCR based gene modification (Longtine et al., 1998). In brief, the *13myc-HisMX6* (for KNY109 and KNY110) and the *13myc-KanMX6* (for KNY111) cassettes encoding the 3'-region of open reading frame and the 3'-UTR region of the *DOA10* gene were made by PCR using pFA6a-13Myc-HisMX6 and pFA6a-13Myc-KanMX6 (Longtine et al., 1998) as a template and primers 5'-GTA AAA GAC GAG GTT TAC ACT AAG GGT AGA GCT TTA GAA AAT TTA CCA GAT GAA AGT CGG ATC CCC GGG TTA ATT AA-3' and 5'-AGA TAT ATA TAT GTA AAT ATG CTA GCA TTC ATT TTA AAT GTA AGG AAG AAA ACG CCT GAA TTC GAG CTC GTT TAA AC -3'. JN516, JB67, and W303-1b were transformed with this fragment to give KNY109, KNY110, and KNY111, respectively. Correct integration was confirmed by PCR using primers 5'-TTC GGA

CTA CTG ATA GCA TTA GA -3' and 5'-GAA CTG AAC GAA AAG TTG GTT GA-3', which localize in the 3'-region of open reading frame and 3'-UTR of *DOA10*, respectively.

The plasmids pSM1082 (*2μ URA3* P_{ste6}*ste6-166*::*HA*), pSM1911 (*2μ URA3* P_{PGK}*ste6-166*::*HA*), pSM1152 (*2μ URA3* P_{PGK}*CFTR*::*HA*), pSM1694 (*2μ URA3* P_{ste6}*ste6-166*::*HA-GFP*), pSM1763 (*CEN URA3 CPY**::*HA*) and YEp105 (*2μ TRP1* P_{CUP1}*mycUb*) were described previously (Huyer et al., 2004; Loayza et al., 1998; Hochstrasser et al., 1991). pRS423mycUb (*2μ HIS3* P_{CUP1}*mycUb*) was constructed as follows. The *BamH*I-*Cla*I fragment containing P_{CUP1}*-mycUb-CYC1* terminator was removed from YEp105 (Hochstrasser et al., 1991) and inserted into the corresponding sites in pRS423 (Christianson et al., 1992) to give pRS423mycUb.

Preparation of Yeast Cytosol and Microsomes

A yeast cytosol S100 fraction (typically 20-30 mg protein/ml) was prepared after centrifugation at 100,000 g by liquid nitrogen lysis from cells grown at 30˚C as described previously (McCracken and Brodsky, 1996). To prepare *SSA1* (JN516), *ssa1-45* (JB67), *YDJ1*/*HLJ1* (W3031b) and *ydj1-151*/*hlj1*Δ cytosol, cells were grown at 26˚C and then shifted to 37˚C for 45 min. To prepare *CDC48* (RSY1168) and *cdc48-3* (RSY1181) cytosol, cells were grown at 26˚C and then shifted to 37˚C for 5 hr (Latterich et al., 1995).

Unless otherwise indicated, microsomes were prepared from 1-2 l of cells by homogenization after spheroplast formation essentially as described (Brodsky et al., 1993). Microsomes from the chaperone mutant and the isogenic wild type cells (Figures 2-4) were prepared by small-scale glass bead disruption. Cells were grown to log phase (OD_{600}=0.7-1.5) and 20-30 OD_{600} equivalents of cells were harvested, washed once with ice-cold distilled water, quick-frozen in liquid nitrogen, and stored at -80°C. The frozen cells were dispersed and disrupted with glass beads added to the meniscus in lysis buffer (20 mM HEPES, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.1 M sorbitol, 1 mM DTT) plus protease inhibitors (1mM PMSF, 1μg/ml leupeptin, and 0.5μg/ml pepstatin A) by

agitation on a Vortex mixer ten times for 30 sec with 30 sec intervals on ice between each cycle. The homogenate was collected and pooled with rinses of the beads with buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc). After unbroken cells were removed by two rounds of centrifugation at 3,000 rpm for 5 min at 4˚C in a refrigerated microcentrifuge, the resulting supernatant was centrifuged at 18,000 g for 20 min at 4˚C. For Figure 6D and Figure S2-B, microsomes were prepared by a medium scale glass bead disruption. The homogenate was prepared from ~200 OD_{600} equivalents of cells by glass bead disruption, layered onto a sucrose cushion (20 mM HEPES, pH 7.4, 50 mM KOAc, 1.0 M sucrose, 1 mM DTT), and centrifuged at 6,500 rpm for 10 min at 4˚C in an HB-6 rotor (Sorvall). The supernatant was collected and centrifuged again at 13,000 rpm for 10 min at 4˚C in an SS-34 rotor (Sorvall). In all three methods, the final membrane pellets were washed, resuspended in buffer 88 to a final concentration of ~10 mg protein/ml (OD_{280} = 40 in 2% SDS), quick-frozen in liquid nitrogen, and stored at -80°C.

***In vitro* Ubiquitination Assay**

Bovine ubiquitin (Sigma #U6253) was dissolved in phosphate buffered saline at a concentration of 10 µg/µl and labeled with ^{125}I (NEN Research, BioRad). The labeled ubiquitin was enriched with a D-salt Excellulose Desalting column (Pierce) and was stored at a final concentration of 0.2 µg/µl (~$1.0x10^6$ cpm/µl). A typical *in vitro* ubiquitination reaction (20 µl) included buffer 88, an ATP regenerating system (McCracken and Brodsky, 1996), 2 µl of microsomes, and the indicated concentration of cytosol, and was pre-warmed at 23˚C (Ste6p*) or at 30˚C (Sec61-2p) for 10 min. After 2 µl of ^{125}I-labeled ubiquitin was added, the reaction was incubated for up to 60 min at the indicated temperatures. The reaction was quenched with 80 µl of 1.25% SDS buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.25% SDS) plus protease inhibitors (1mM PMSF, 1µg/ml leupeptin, and 0.5µg/ml pepstatin A) and 10 mM *N*-ethylmaleimide. After incubation at 37˚C for 30 min, the samples were mixed with 400 µl of 2% TritonX-100 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% TritonX-100)

plus protease inhibitors and 10 mM *N*-ethylmaleimide, kept on ice, and then incubated with 10 µg of anti-HA antibody (Roche) or 3µl of anti-Sec61p antiserum (Stirling et al., 1992) at 4˚C for ~16 hr. A total of 30 µl of pre-equilibrated 50% (v/v) Protein A-Sepharose (GE Healthcare) was added and the mixture was incubated at 4˚C for 2-4 hr. The immunoprecipitates were washed 4 times with IP wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 0.2% SDS) plus 10 mM *N*-ethylmaleimide, and bound material was eluted with 30 µl of either 2x SDS-PAGE sample buffer (4% β-mercaptoethanol, 4% SDS, 130 mM Tris pH6.8, 20% glycerol, 10 mg/ml Bromophenol blue) or TCA sample buffer (80 mM Tris pH 8.0, 8 mM EDTA, 0.12 M-0.5 M DTT, 3.5% SDS, 15% glycerol, 0.08% Tris-base, 0.01% Bromophenol blue) at 37˚C for 30 min. Proteins were resolved on 6% or 10% SDS-polyacrylamide gels. Ubiquitinated species were detected by phosphor imager analysis using Image Gauge Software (v3.45; Fuji Film Science Lab). Unmodified proteins were detected by western blot analysis using anti-HA antibody or anti-Sec61p antiserum, a horseradish peroxidase-conjugated secondary antiserum, and a Kodak 440CF Image Station and the associated Kodak 1D (V. 3.6) software (Rochester, NY). When the extraction of a ubiquitinated protein was assessed, the completed *in vitro* reactions were centrifuged at 18,000 g for 10 min at 4˚C, and the supernatant (S) and pellet (P) fractions were subjected to an immunoprecipitation, as described above.

To detect the effect of purified Ssa1p and Ydj1p (prepared as described in McClellan and Brodsky, 2000; Cyr et al., 1992; Caplan et al., 1992) on in vitro ubiquitination, wild type (KNY100) microsomes (~25 µg) were first incubated with the indicated amount of Ssa1p and Ydj1p, and an ATP regenerating system at 23˚C for 5 min and then at 37˚C for 15 min. As a control, BSA was included at the same concentration. Subsequently, ^{125}I-labeled ubiquitin and wild type (RSY607) cytosol (at a final concentration of 1 µg/µl) were added and the reaction was further incubated at 23˚C for 40 min before the samples were processed as described above.

To detect the interaction between polyubiquitinated Ste6p* and TAP-tagged Cdc48p, a 250 µl *in vitro* reaction was performed in the presence of protease inhibitors,

and the reaction was centrifuged as described above. The supernatant (170 µl) was added to 340 µl of buffer 88 and incubated with 30 µl of 50% (v/v) buffer 88-equilibrated IgG sepharose (GE Healthcare) for 3 hr at 4 ˚C. The immunoprecipitates were washed two times with 900 µl of buffer 88, one time with 900 µl of buffer 88 plus 0.2% TritonX-100, and one time with 900 µl of buffer 88. The bound materials were eluted with 100 µl of 1% SDS buffer plus protease inhibitors and 10 mM *N*-ethylmaleimide at 37˚C for 30 min, and subjected to the second round of immunoprecipitation with anti-HA antibody. The immunoprecipitates were resolved on SDS-polyacrylamide gels and analyzed as above.

To detect the degradation of cytosolic ubiquitinated Ste6p*, the supernatant after the *in vitro* assay was enriched 5-10 fold with a YM-100 membrane filter (Amicon). The reaction (20 µl) included the enriched cytosol containing ubiquitinated Ste6p*, 11 µl of untreated rabbit reticulocyte lysate (Promega, L#4151), an ATP regenerating system (1 mM ATP, 10 mM creatine phosphate, 100 µg/ml creatine phosphokinase), in 20 mM Tris pH7.5, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol. Where indicated, the rabbit reticulocyte lysate was preincubated with 1% DMSO, or 125 µM MG132 and 90 µM epoxomicin on ice for 90 min. The reaction was performed at 30˚C for the indicated times and quenched by 80 µl of 2.5% SDS buffer plus protease inhibitors and 10 mM *N*-ethylmaleimide. After incubation at 37˚C for 30 min, the samples were mixed with 900 µl of 1% TritonX-100 buffer plus protease inhibitors and 10 mM *N*-ethylmaleimide, and Ste6p*HA was immunoprecipitated with anti-HA antibody as above. The immunoprecipitates were resolved on SDS-polyacrylamide gels and analyzed as above.

Cross-linking Assay

A total of 20-30 OD_{600} equivalents of log phase cells (~OD_{600}=1.0) were collected at each time point, washed with ice cold distilled water, quick frozen in liquid nitrogen, and stored in –80˚C. Microsomes were prepared by glass bead disruption (see above) using buffer 88 at pH 7.5 instead of pH 6.8. Next, 250 µg of microsomes were incubated with freshly prepared 100 µg/ml of Dithiobis (succinimidyl) propionate (DSP)

(Pierce) or 100 µg/ml of 3,3-Dithiobis[sulfosuccinimidylpropionate] (DTSSP) in 100 µl of buffer 88 (pH 7.5) for 60 min on ice. The reaction was quenched with 30 µl of 1 M Tris pH 7.5 and the solution was incubated for 20 min. The microsomes were collected by centrifugation, washed once with 1 ml of buffer 88 (pH 7.5) supplemented with 0.2 M TrisCl (pH7.5), and resuspended in 100 µl of 1% SDS buffer and incubated at 37˚C for 30 min. The detergent-treated samples were added to 400 µl of 2% TritonX-100 buffer or 900 µl of 1% TritonX-100 buffer, and were cleared by centrifugation at 4,500 rpm for 5 min in a refrigerated microcentrifuge at 4˚C after the solution was pre-cleaned by incubation with 20 µl of 50 % (v/v) in Tris-buffered saline-equilibrated Protein A-Sepharose for 1.5 hr at 4˚C. The final supernatant was mixed with 10 µg of anti-HA antibody (Roche) and incubated at 4˚C for ~16 hr. Then, 30 µl of 50% (v/v) pre-equilibrated Protein A-Sepharose was added and the mixture was incubated for 2 hr at 4˚C. Alternatively, the immunoprecipitation was performed using 20 µl of 50% (v/v) anti-HA affinity matrix (Roche) by incubation with pre-cleared lysate using unconjugated Sepharose 6B at 4˚C for ~3 hr. In either case, the immunoprecipitates were washed four times with ice-cold IP wash buffer and the bound proteins were eluted and the cross-linker was cleaved with 30 µl of TCA sample buffer at 37˚C for 20 min and then at 42˚C for 10 min. Proteins were resolved by SDS-PAGE and the indicated proteins were detected by western blot analysis with anti-HA antibody or anti-myc antibody (a kind gift from O. Weisz and G. Apodaca, University of Pittsburgh School of Medicine).

Co-Immunoprecipitation

A total of 75 OD_{600} equivalents of log phase cells (~OD_{600}=1.0) expressing Ste6p*-HA and Doa10-13myc were collected after the temperature was shifted from 23˚C to 37˚C for 1 hr. The cells were then washed with ice cold distilled water, quick frozen in liquid nitrogen, and stored in –80˚C. Next, the cells were disrupted with glass beads, as described above, but instead a buffer containing 50 mM Tris-Cl pH 7.4, 100 mM NaCl, 5 mM EDTA, and 200 mM sorbitol plus protease inhibitors (1mM PMSF, 1µg/ml leupeptin, and 0.5µg/ml pepstatin A) was used. After the cell debris and

unbroken cells were removed by centrifugation, for native precipitations, the supernatant (~2.7 ml) was mixed with Triton X-100 at a final concentration of 1% and the solution was incubated on ice for 30 min. The detergent solubilized lysate was cleared by ultracentrifugation at 30,000 g for 15 min at 4˚C, and the supernatant was pre-cleared with pre-equilibrated Sepharose 6B, as described above but for 20 min. To measure the input/lysate, ~20 µl of the supernatant was saved at this point. Then, the remaining supernatant was incubated with 30 µl of 50% (v/v) anti-HA affinity matrix at 4˚C for 1 hr with rocking. The matrix was washed with 6 times with 2.7 ml of ice-cold buffer (50 mM Tris-Cl pH 7.4, 100 mM NaCl, 5 mM EDTA, 200 mM sorbitol, 1% Triton X-100) and the bound proteins were eluted with SDS-PAGE sample buffer (see above). For denaturing precipitations, cells were disrupted in the presence of 1% SDS and heated at 37°C for 5 min before the lysate was diluted into the buffer described above such that the final SDS concentration was 0.2%. After the cell debris was removed, the samples were solubilized with TritonX-100 and centrifuged as described above. Samples were washed in the presence of 0.2% SDS twice with the same wash buffer above, and then twice with urea-containing buffer, once with high salt-containing buffer, and once with the wash buffer.

Purification of GST-Tagged Ufd2p

A plasmid encoding a GST-Ufd2p-myc fusion protein was expressed in BL21 DE3 cells (Kim et al., 2004; a kind gift from H. Rao, University of Texas Health Sciences Center, San Antonio). The expression of the protein was induced for 4 hr at 26˚C with 0.5 mM isopropyl β-D-thiogalactosidase. The cells were then suspended in phosphate buffered saline (PBS) containing 1% Triton X-100 and lysed by sonication. The extract was centrifuged at 10,000 rpm for 10 min at 4˚C in a Sorvall SS-34 rotor. Next, the supernatant was incubated with 1.3 ml of 50% (v/v) Glutathione Sepharose for 3 hr at 4˚C, the resin was packed into a column, and the column was washed with 15 ml of PBS containing 1% Triton X-100 and then with 15 ml of PBS. The bound protein was eluted with PBS containing freshly-dissolved 10 mM glutathione, dialyzed against buffer 88,

and enriched using Centricon 30 and YM-100 membrane filters (Amicon). The GST control protein, purified in the similar manner, was a kind gift from R.T. Youker (prepared as described in Youker et al., 2004) and the buffer was exchanged with buffer 88 using a NAP-10 gel filtration column (Amersham Pharmacia Biotech).

Cycloheximide Chase Degradation Assay

Cells were grown for ~16 h to log phase (OD_{600}=~0.8), harvested and resuspended in fresh medium at a concentration of 1.0 OD_{600}/ml. Cycloheximide (CHX) (50-250 µg/ml) was added and 1 ml of culture was taken at the indicated time points. This aliquot was first added to 30 µl of 0.5 M NaN_3 on ice and cells were collected at 10,000 g for 2 min at 4˚C. The pelleted cells were then washed with 800 µl of ice-cold 10 mM NaN_3, collected again as above, snap-frozen in liquid nitrogen, and stored at –80˚C. The frozen cells were dispersed and lysed in 85 µl of 10 mM Tris pH 7.5, 1 mM EDTA, supplemented with 7 µl of 5N NaOH, 6.3 µl of 14.4 M β-mercaptoethanol and the protease inhibitors described above. After incubation for 20 min on ice, 1 ml of 10% TCA was added and the incubation was continued for 20 min on ice. Proteins were precipitated by centrifugation at 18,000 g for 5 min at 4˚C, and the pellet was rinsed with 800 µl of ice-cold acetone and dissolved in 60-80 µl of TCA sample buffer. Samples were heated at 37 ˚C for 30 min and insoluble material was removed by centrifugation at 12,000 g for 2 min at room temperature. Proteins were resolved by SDS-PAGE and analyzed by western blot using the indicated antisera.

Figure S1

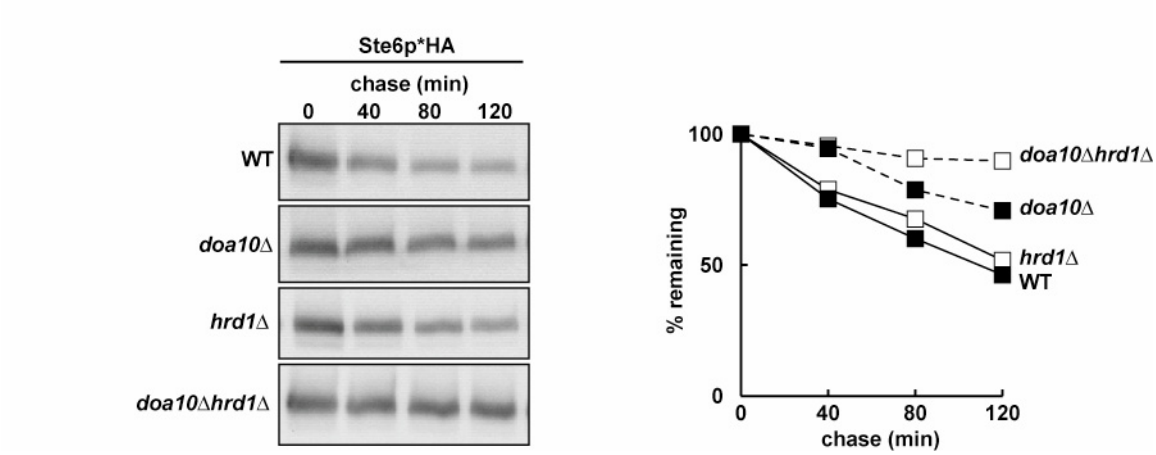

Figure S1. Hrd1p can partially compensate for Ste6p*HA degradation in the absence of Doa10p.

The degradation of Ste6p*HA in wild-type (KNY100), *doa10*Δ (KNY108), *hrd1*Δ (KNY102) and *doa10*Δ/*hrd1*Δ (KNY103) cells was analyzed by immunoblotting with anti-HA antibody following inhibition of translation with cycloheximide. Cells were transformed with pSM1911 and grown at 30˚C. Similar results were previously reported (Huyer et al., 2004).

Figure S2

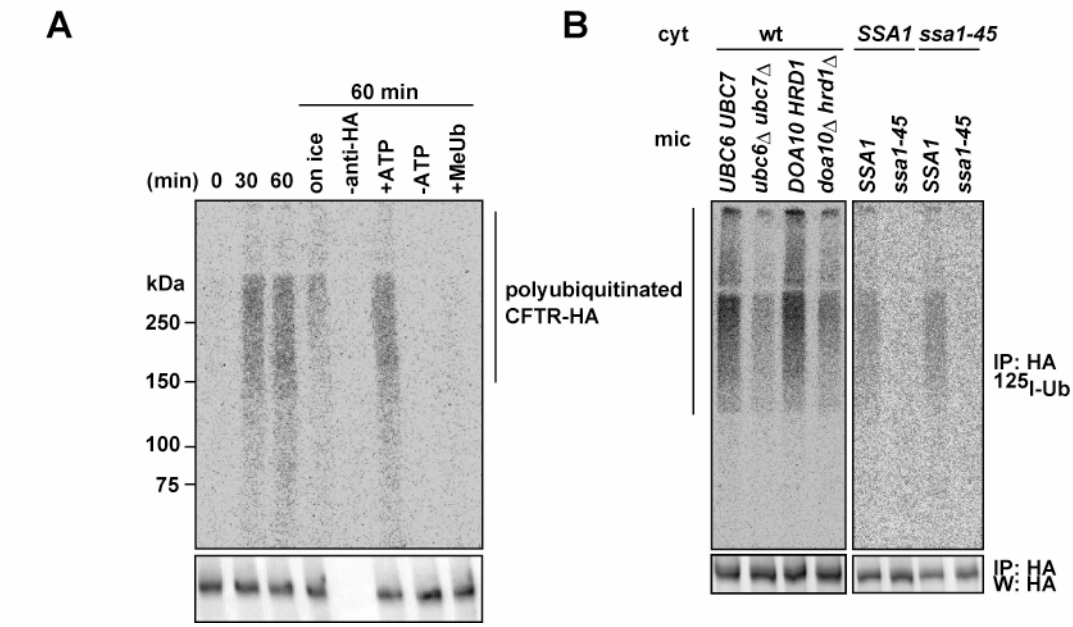

Figure S2. ***In vitro*** **polyubiquitination of CFTR.**

(A) An *in vitro* ubiquitination assay was performed for CFTR using the protocol described in Figure 1 and above. Polyubiquitinated CFTR and unmodified CFTR were detected by phosphorimager (top) and western blot analysis (bottom), respectively. Microsomes were prepared from wild type (KNY114) cells transformed with pSM1152 (CFTR-HA) grown at 30˚C. Cytosol was prepared from wild type (RSY607) cells.

(B) Microsomes were prepared by glass bead disruption from *UBC6/UBC7* (RHY501), *ubc6*Δ/*ubc7*Δ (RHY505), *HRD1*/*DOA10* (KNY100) and *hrd1*Δ/*doa10*Δ (KNY103) cells expressing CFTR as in (A) grown at 30˚C, or from *SSA1* (JN516) and *ssa1-45* (JB67) cells expressing CFTR grown at 23˚C and then shifted to 37˚C for 90 min. Cytosol was prepared from wild type (RSY607), *SSA1* (JN516) and *ssa1-45* (JB67) cells. Cyt, cytosol; mic, microsomes.

Figure S3

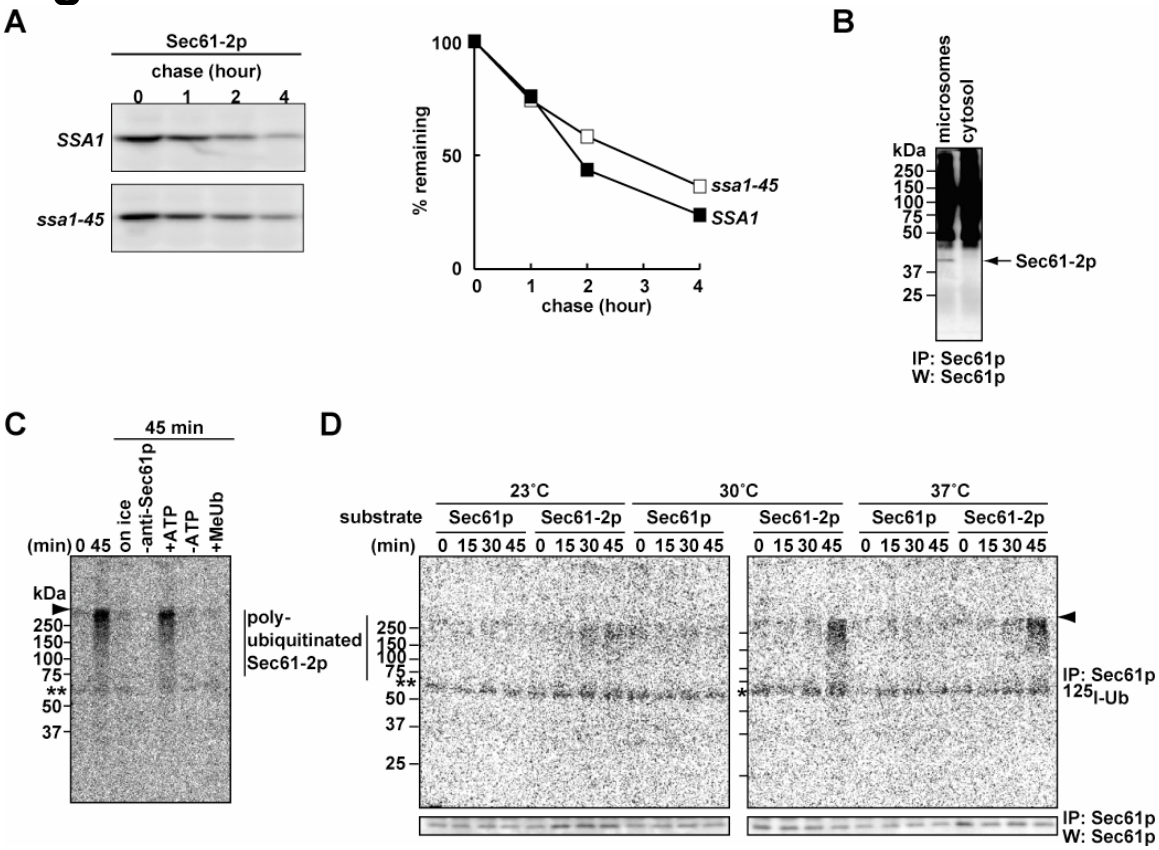

Figure S3. Cycloheximide chase analysis and *in vitro* polyubiquitination of Sec61-2p.

(A) The cytoplasmic Hsp70, Ssa1p, plays only a minor role during the degradation of Sec61-2p *in vivo*. The degradation of Sec61-2p in *SSA1* and *ssa1-45* cells was analyzed. Cells were grown at 23˚C and then shifted to 37˚C for 45 min before translation was inhibited with cycloheximide. Next, the cells were collected at the indicated time points and Sec61-2p was detected by immunoblotting with anti-Sec61p antiserum. The strains used were SNY1077 (*sec61-2*/*SSA1*) and SNY1078 (*sec61-2*/*ssa1-45*) (Nishikawa et al., 2001).

(B) Immunoprecipitation of Sec61-2p with anti-Sec61p antibodies. Microsomes prepared from *sec61-2* (RSY533) cells and cytosol prepared from wild type (RSY1168) cells were subjected to immunoprecipitation with anti-Sec61p antiserum. The immunoprecipitates were analyzed by western blotting with anti-Sec61p antiserum.

(C) Sec61-2p is ubiquitinated *in vitro*. Microsomes were prepared from *sec61-2* (RSY533) cells grown at 23˚C, and the requirements for the ubiquitination of Sec61-2p were analyzed as described for Ste6p* in Figure 1 and above.

(D) The *in vitro* ubiquitination of Sec61p and Sec61-2p were analyzed at 23˚C, 30˚C and 37˚C. Microsomes were prepared from *SEC61* (RSY156) and *sec61-2* (RSY533) cells grown at 23˚C, and were subjected to the *in vitro* assay for the indicated times and at the indicated temperatures. The double asterisks show proteins which contaminated bovine ubiquitin and nonspecifically cross reacts with anti-Sec61p antiserum, and thus appear at the 0 min independent of ATP and temperature. Arrowhead indicates the boundary between the 4% stacking gel and the 10% resolving gel.

Figure S4

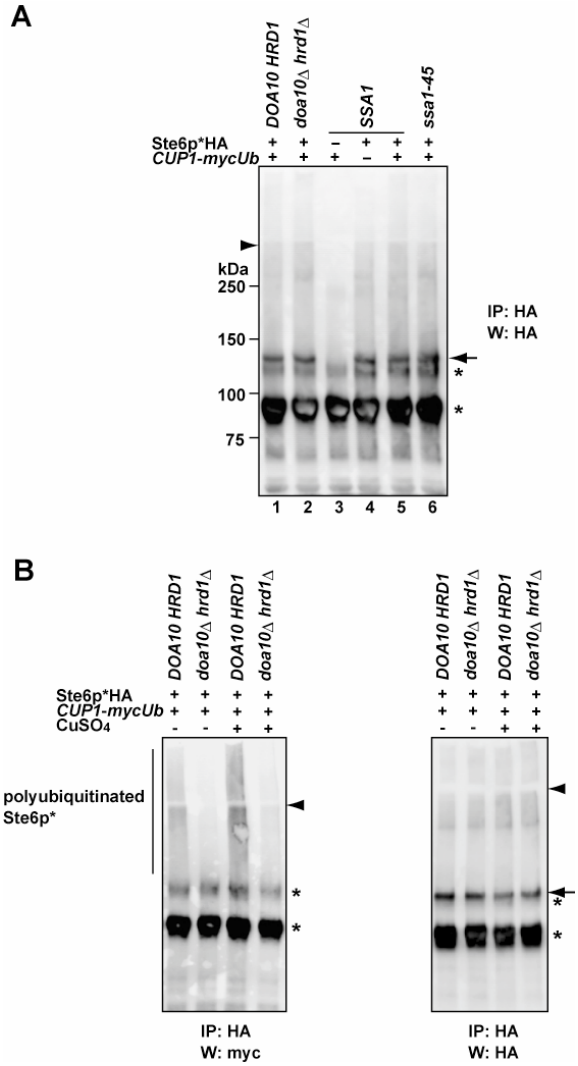

Figure S4. ***In vivo*** **analysis of of Ste6p*.**

(A) Full size blot for the bottom panel of Figure 2D (IP: HA, W: HA). The arrow indicates Ste6p*HA and the asterisks indicate the positions of IgG. The arrowhead indicates the boundary between 4% stacking gel and the 6% resolving gel. Please note that the apparent "smear" in this figure does not represent polyubiquitinated material since it is apparent in extracts from *doa10Δhrd1Δ* yeast (lane 2) and does not react with anti-ubiquitin antiserum (Figure 2D).

(B) Ste6p* polyubiquitination *in vivo* was analyzed in E3 ligase mutant cells as in Figure 2D through the use of affinity purified anti-myc monoclonal antibody (9E10, a gift from

G. Apodaca, University of Pittsburgh School of Medicine). Note that the IgG complex is incompletely dissociated because protein samples were incubated at 37˚C for 30 min in SDS-PAGE sample buffer.

Figure S5

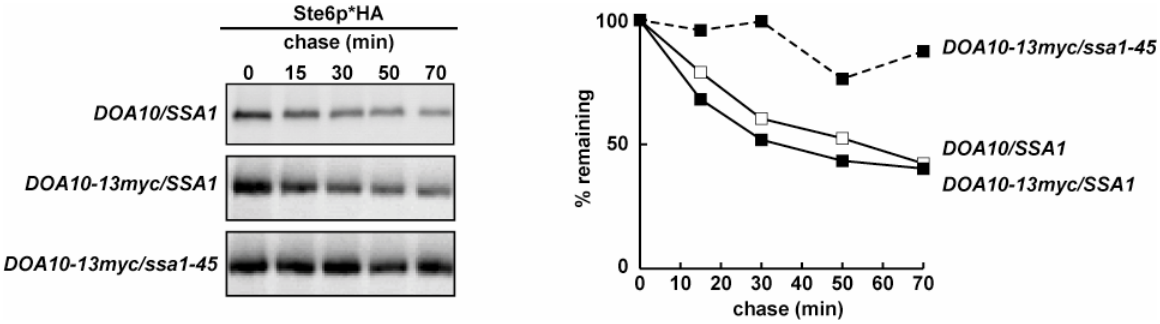

Figure S5. The 13myc-tagged form of Doa10p supports Ste6p* ERAD *in vivo*.

The degradation of Ste6p*HA in *DOA10*/*SSA1* (JN516), *DOA10-13myc*/*SSA1* (KNY109), and *DOA10-13myc*/*ssa1-45* (KNY110) cells was analyzed by western blotting with anti-HA antibody following inhibition of translation with cycloheximide. Cells were grown at 26˚C and shifted to 37˚C for 10 min before cycloheximide was added.

Figure S6

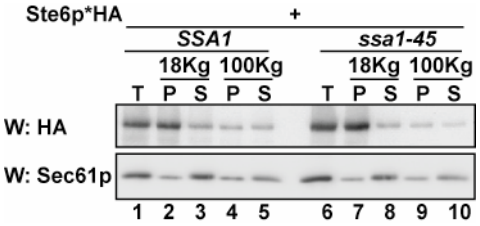

Figure S6. Detergent solubility of Ste6p* in Hsp70 mutant cells.

SSA1 (JN516) and *ssa1-45* (JB67) cells expressing Ste6p*HA were incubated at 23˚C and shifted to 37˚C for 45 min. Microsomes were prepared by glass bead disruption, solubilized with 1% TritonX-100, and the solution was centrifuged at 18,000 g. The supernatant was collected and centrifuged at 100,000 g. Ste6p*HA and Sec61p, as a control, were identified by western blot analysis.

Figure S7

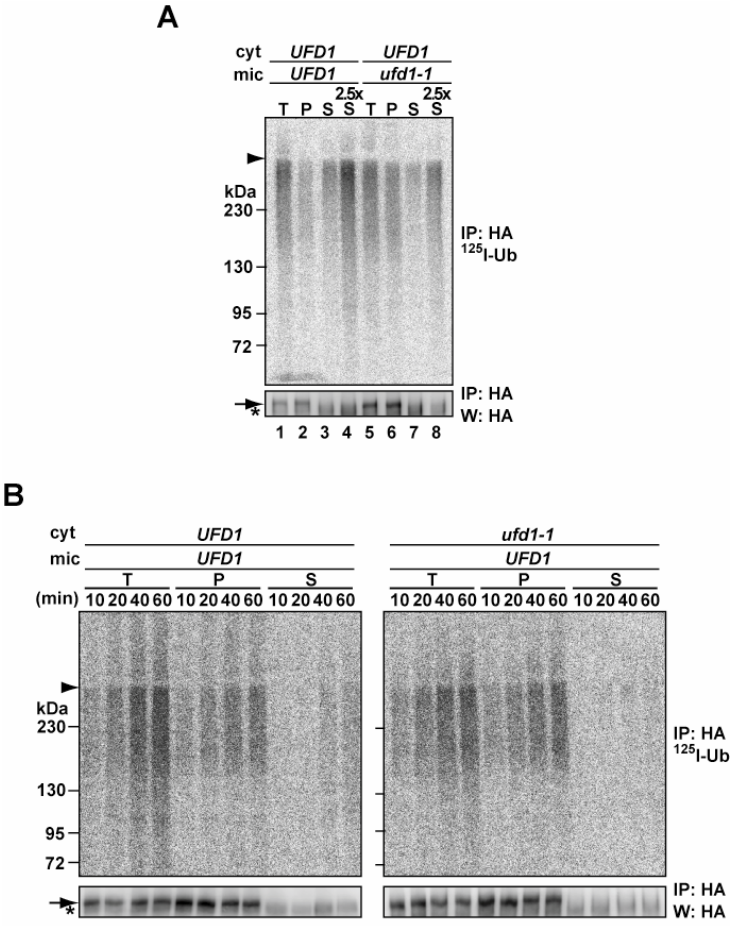

Figure S7. Ufd1p is required for maximal release of polyubiquitinated Ste6p* from the ER membrane.

(A) An *in vitro* ubiquitination and supernatant/pellet assay were performed as described in Figure 6 using microsomes prepared from *UFD1* (Bwg1-7a) or *ufd1-1* (PM373) cells expressing Ste6p*HA grown at 26˚C and shifted to 37˚C for 2.5 h, and cytosol prepared from wild type (RSY1168) cells. In lanes 4 and 8, a 2.5-fold increased volume of supernatant was subjected to immunoprecipitation with anti-HA to better resolve the distinct release of polyubiquitinated material. The arrowhead indicates the boundary between the 4% stacking gel and the 6% resolving gel. The arrow indicates Ste6p*HA, and the asterisk indicates the positions of IgG. Cyt; cytosol, mic; microsomes.

(B) An *in vitro* ubiquitination assay was performed for the indicated times using cytosol from *UFD1* (Bwg1-7a) and *ufd1-1* (PM373) cells, and microsomes from wild type (W303-1b) cells expressing Ste6p*HA. After the reaction, microsomes and cytosol were separated by centrifugation and each fraction was subjected to immunoprecipitation

with anti-HA antibody.

Figure S8

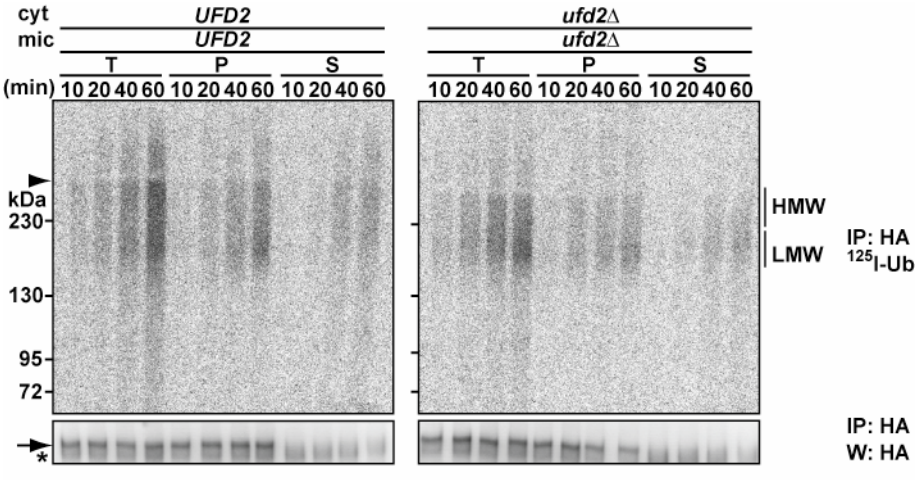

Figure S8. Ufd2p is dispensable for the release of ubiquitinated Ste6p*.

An *in vitro* ubiquitination assay was performed for the indicated times using cytosol from *UFD2* (BY4741) and *ufd2* (BY3888) yeast, and microsomes from *UFD2* (BY4741) and *ufd2* (BY3888) cells expressing Ste6p*HA. After the reaction, microsomes and cytosol were separated by centrifugation and each fraction was subjected to immunoprecipitation with anti-HA antibody. The arrow indicates Ste6p*HA, and the asterisk indicates the position of IgG. Cyt, cytosol; mic, microsomes.

Figure S9

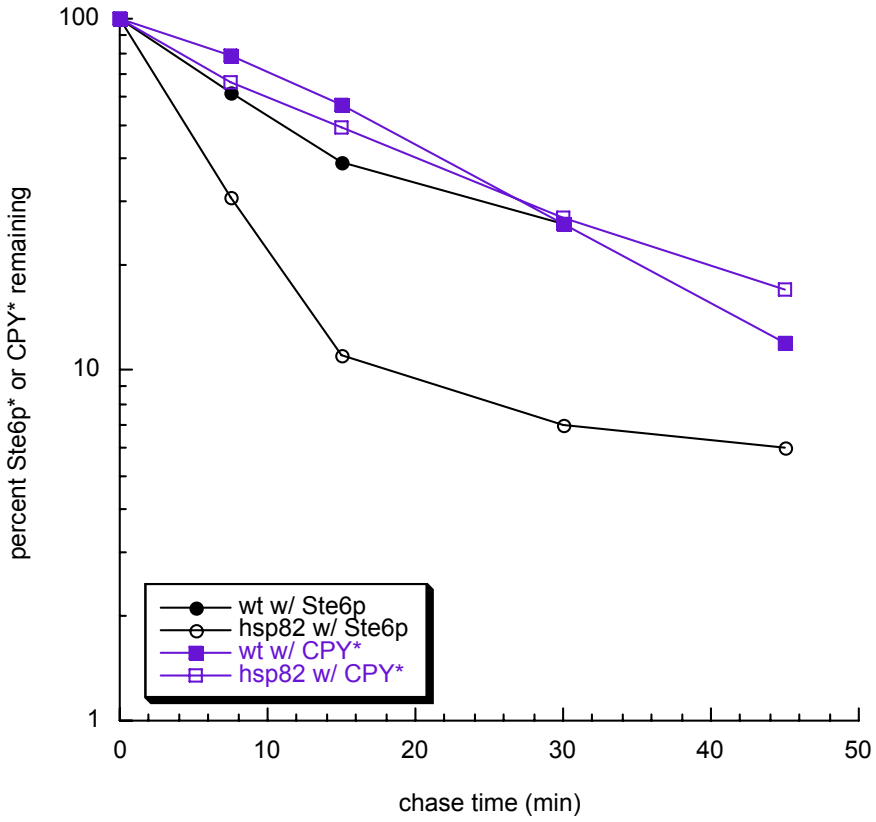

Figure S9. Depletion of Hsp90 (Hsp82 in yeast) accelerates the degradation of Ste6p*.

The degradation of Ste6p*HA-GFP (pSM1694) and CPY* (pSM1763) in wild type (SM4552) and *hsp82* mutant (SM4553) cells were analyzed by pulse chase analysis as described in Huyer et al., 2004. Before labeling, cells were shifted to 37˚C for 20 min.

Table S1

Strains used in this study

Strain	Relevant genotype	Ref./source
Bwg1-7a	*MAT***a** *his4-519 ura3-52 ade1-100 leu2-3,112*	Johnson et al., 1995
BY4741	*MAT***a** *his3 leu2 met15 ura3*	H. Rao
BY3888	*MAT***a** *his3 leu2 met15 ura3 ufd2::kanMX*	H. Rao
JN516	*MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2*	Becker et al., 1996
JB67	*MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1-45 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2*	Becker et al., 1996
KNY100	*MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*	M. Hochstrasser
KNY102	*MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 HRD1::LEU2*	M. Hochstrasser
KNY103	*MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa10::HIS3 HRD1::LEU2*	M. Hochstrasser
KNY108	*MATα his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1 doa10::kanMX*	This study
KNY109	*MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2 DOA10-13MYC-HIS3MX*	This study
KNY110	*MATα his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1-45 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2 DOA10-13MYC-HIS3MX*	This study
KNY114	*MAT***a** *ade2 his3 leu2 ura3 trp1*	Youker et al., 2004
KNY115	*MAT***a** *his3 leu2 met15 ura3 CDC48-TAP-HIS*	Open Biosystems
KNY111	*MATα ade2 his3 leu2 ura3 trp1 can1-100 DOA10-13MYC-KANMX*	This study
PM373	*MAT***a** *his4-519 ura3-52 ade1-100 leu2-3,112 ufd1-1*	Johnson et al., 1995
RHY501	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*	R. Hampton
RHY505	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ubc6::HIS3 ubc7::LEU2*	R. Hampton
RSY156	*MATα leu2-3,112 ura3-52 pep4-3*	R. Schekman
RSY533	*MATα leu2-3,112 ura3-52 pep4-3 ade2 sec61-2*	R. Schekman

RSY607	*MATα ura3-52 leu2-3112 pep4::URA3*	R. Schekman
RSY1168	*MAT***a** *ura3-52 leu2,3-112 pep4::URA3*	Latterich et al., 1995
RSY1181	*MAT***a** *ura3-52 leu2,3-112 pep4::URA3 cdc48-3*	Latterich et al., 1995
SM4552	*MAT***a** *ade2-1 leu2-3, 112 his3-11, 15 trp1-1 ura3-1 can1-100 hsc82::LEU2 hsp82::LEU2 pTGPD-HSP82*	Nathan and Lindquist, 1995
SM4553	*MAT***a** *ade2-1 leu2-3, 112 his3-11, 15 trp1-1 ura3-1 can1-100 hsc82::LEU2 hsp82::LEU2 pTGPD-HSP82-G313N*	Nathan and Lindquist, 1995
SNY1077	*MATα sec61-2 his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2*	Nishikawa et al., 2001
SNY1078	*MATα sec61-2 his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1-45 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2*	Nishikawa et al., 2001
W3031b	*MATα ade2 his3 leu2 ura3 trp1 can1-100*	Caplan et al., 1992
ydj1-151/hlj1Δ	*MATα ade2 his3 leu2 ura3 trp1 can1-100 ydj1-2::HIS3 ydj1-151::LEU2 hlj1::TRP1*	Youker et al., 2004

Supplemental References

Becker, J., Walter, W., Yan, W. and Craig, E. A. (1996). Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. Mol Cell Biol *16*, 4378-4386.

Brachmann, C.B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132.

Brodsky, J. L., Hamamoto, S., Feldheim, D. and Schekman, R. (1993). Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic Hsc70. J Cell Biol *120*, 95-102.

Caplan, A.J., Tsai, J., Casey, P.J., and Douglas, M.G. (1992). Farnesylation of YDJ1p is required for function at elevated growth temperatures in Saccharomyces cerevisiae. J Biol Chem *267*, 18890-18895.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119-122.

Cyr, D.M., Lu, X., and Douglas, M.G., (1992). Regulation of Hsp70 function by a eukaryotic DnaJ homolog. J Biol Chem *267*, 20927-20931.

Hochstrasser, M., Ellison, M. J., Chau, V., Varshavsky, A. (1991). The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. Proc Natl Acad Sci U S A *88*, 4606-4610.

Huyer, G., Piluek, W. F., Fansler, Z., Kreft, S. G., Hochstrasser, M., Brodsky, J. L. and Michaelis, S. (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. J Biol Chem *279*, 38369-38378.

Kim, I., Mi, K., and Rao, H. (2004). Multiple interactions of Rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. Mol Biol Cell *15*, 3357-3365.

Latterich, M., Frohlich, K. U., and Schekman, R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell *82*, 885-893.

Loayza, D., Tam, A., Schmidt, W. K., and 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*, 2767-2784.

Longtine, M.S., Mckenzie III, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

McClellan, A.J., and Brodsky J.L. (2000). Mutation of the ATP-Binding Pocket of *SSA1* Indicates That a Functional Interaction Between Ssa1p and Ydj1p Is Required for Post-translational Translocation Into the Yeast Endoplasmic Reticulum. Genetics *156*, 501-512.

McCracken, A. A. and Brodsky, J. L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. J Cell Biol *132*, 291-298.

Nathan, D. F., and Lindquist, S. (1995). Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase. Mol. Cell. Biol. *15,* 3917-3925.

Nishikawa, S. I., Fewell, S. W., Kato, Y., Brodsky, J. L. and Endo, T. (2001). Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol *153*, 1061-1070.

Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995). A proteolytic pathway that recognizes ubiquitin as a degradation signal. J Biol Chem *270*, 17442-17456.

Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R., and Schekman, R. (1992). Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. Mol Biol Cell *3*, 129-142.

Youker, R.T., Walsh, P., Beilharz, T., Lithgow, T., and Brodsky J.L. (2004). Distinct Roles for the Hsp40 and Hsp90 Molecular Chaperones during Cystic Fibrosis Transmembrane Conductance Regulator Degradation in Yeast. Mol Biol Cell *15*, 4787-4797.