Extended Experimental Procedures

Strains

All deletion strains used in this study were purchased from GE Dharmacon. Single deletions are derivatives of BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). CPY*-TM, CPY-TM, CPY*-TM2 and CPY-TM2 were expressed in a *hrd3Δalg3Δ* strain derived from BY4741 (yAST-26 - MATa *ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 hrd3::KANR alg3::HIS3*). Hrd1 and its variants were expressed in a homozygous diploid *ubc7Δ* strain (MATa/MATα *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 ubc7::KanR/ubc7::KanR*). For expression in *E. coli*, BL21-CodonPlus (DE3)-RIPL was used (Stratagene).

Purification of proteins

Uba1, Cue1ΔTM, and Ubc7 were purified as described (Stein et al., 2014).

CPY*-TM, CPY-TM, CPY*-TM2, CPY-TM2, or CPY*-TM-noK were expressed in *S. cerevisiae*. Yeast cells were transformed with the expression plasmid using the Lithium-acetate method (Gietz and Schiestl, 2007). A starter culture was inoculated and grown overnight at 30°C in synthetic dropout medium with amino acid supplements and 2% glucose. This culture was diluted 1:100 into fresh medium and grown for 24 hrs at 30°C. Expression was induced by adding induction media (4x YP with 8% galactose) at a ratio of 1:3, induction media : culture (final 1x YP, 2% galactose). The culture was grown for an additional 16-18 hrs at 30°C, or at 25°C for Hrd1. Cells were pelleted at 4000 x g, washed once in water, and stored at -80°C.

To prepare cell lysates, ~100 g of yeast cells were resuspended in 200 ml of buffer A (50mM HEPES pH 7.4, 300mM KCl). Immediately before lysis, the buffer was supplemented with 1mM phenylmethanesulfonyl fluoride (PMSF) and 1.5mM pepstatin A. Glass beads were added to 1/3 the volume of the cell suspension. Cells were lysed in a BioSpec BeadBeater for 30 min with 30sec/60 sec on/off cycles. After removal of the glass beads, the lysate was centrifuged twice at 2,000 x g for 10 min. The supernatant was centrifuged in a Ti45 rotor at 42,000 rpm (RCF_{avg} 138,000) for 30 min at 4°C. The membrane fraction was resuspended and pelleted twice. It was flash-frozen in liquid N_2, and stored at -80°C.

The membrane fraction was resuspended in approximately 200ml of buffer S_{UFI} (50mM HEPES, pH 7.4, 300mM KCl, 1mM $MgCl_2$, 1mM tris (2-carboxyethyl)phosphine (TCEP), 6M urea, 1% tridecylphosphocholine (Fos-choline 13, Fos13), 30mM imidazole) for 60 min at 4°C. Insoluble material was removed by centrifugation in a Ti45 rotor (42,000 rpm, 30 min, 4°C). The supernatant was incubated at 4°C for 60 min with His60 Ni Superflow resin (Clontech). The resin was washed with 10 column volumes (CV) of buffer B_{UFI} (25mM HEPES, pH 7.4, 300mM KCl, 1mM $MgCl_2$, 0.5mM TCEP, 6M urea, 2mM Fos13, 30mM imidazole) at 4°C. The column was washed with 10CV B_{UFI} (5M urea), 10CV B_{UFI} (3M urea), 10 CV B_{UFI} (1M urea), 20CV B_{FI} (no urea). The protein was then eluted with buffer B_{FI} (400mM imidazole), labeled with fluorescent dyes either by sortase at the C-terminus or on amines, and purified by gel filtration. To purify CPY-TM and CPY-TM2, the proteins were tagged with a KKTN ER retention signal on their C-terminus (Gaynor et al., 1994). Sortase-labeling replaced this signal with a fluorescent peptide.

To purify Hrd1 and its variants, the crude membrane fraction was resuspended in approximately 200ml of buffer S_D (50mM HEPES, 300mM KCl, 1mM $MgCl_2$, 1mM TCEP, 1% decyl maltose neopentyl glycol (DMNG)) and incubated for 60 min at 4°C. Insoluble material was removed by centrifugation in a Ti45 rotor (42,000 rpm, 30 min, 4°C) and the supernatant was incubated with high-capacity streptavidin agarose resin (Pierce) at 4°C for 3 hrs. The resin was washed with 20CV of buffer B_D (25mM HEPES, 300mM KCl, 1mM $MgCl_2$, 0.5mM TCEP, 120 μM DMNG), then 20CV of buffer B_D +0.5mM ATP at room temperature, and finally 100CV of buffer B_D at 4°C. The protein was then eluted with buffer B_D +2mM biotin. After elution, Hrd1 was labeled with fluorescent dyes either by sortase at the C-terminus or at amines with an NHS-ester. The protein was purified by size exclusion chromatography on a Superose 6 10/300 GL equilibrated with buffer B_D.

Rsp5 was expressed in *E. coli* with an N-terminal His_{14}-SUMO fusion (Frey and Gorlich, 2014). The cells were grown at 37°C to a density of 1 OD_{600}/mL in terrific broth, and recombinant protein expression was induced with 500μM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C for 16 hrs. Cells were pelleted at 4,000 x g and resuspended in buffer C_I (50mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole), supplemented with PMSF and protease inhibitor cocktail. Cells were lysed using a microfluidizer M110-P (Microfluidics) with two passes at 18,000 PSI.

The lysate was cleared by centrifugation in a Ti45 rotor (42,000 rpm, 30 min, 4°C) and the lysate was incubated with His60 Ni Superflow resin for 1.5 hours at 4°C. The resin was washed with 50CV of buffer C_I and eluted with buffer C +400mM imidazole. Immediately after elution, glycerol was added to 10% and TCEP to 0.5mM. To remove the His_{14}-SUMO tag, SUMO protease was added to 1μM and incubated at 4°C for 3 hrs. Following cleavage, the protein was dialyzed against 50mM HEPES, pH 7.4, 300mM KCl, 0.5mM TCEP, 10% glycerol. After passage through His60 Ni Superflow resin to remove the His_{14}-SUMO tag and the His-tagged protease, the protein was loaded onto a MonoQ 10/100 GL column equilibrated with the same buffer. The protein was eluted by a linear gradient to 500mM KCl over 20CV. Peak fractions were pooled.

Labeling with fluorescent dyes

ERAD substrates and Hrd1 were sortase-labeled at their C-termini using DyLight680, DyLight800, or Alexa488 maleimide conjugated to a peptide with the sequence Gly-Gly-Gly-Cys. The sortase A variant carrying five point mutations (P94R/D160N/D165A/K190E/K196T) was purified from *E. coli* (Chen et al., 2011). For labeling, 80 μM CPY*-TM variants (or 20μM Hrd1) was incubated with 3 μM sortase, and 500 μM labeled peptide in sortase buffer (50mM HEPES, 300mM KCl, 1mM $MgCl_2$, 0.5mM TCEP, 10mM $CaCl_2$), and 2mM Fos-13 (for CPY*-TM variants) or 120 μM DMNG (for Hrd1), for 16 hrs at 4°C. The labeling efficiency was 75-90% for CPY*-TM variants and ~50% for Hrd1.

Free amines in substrates were labeled with AlexaFluor488-tetrafluorophenyl ester (Alexa488-TFP; Life Technologies) or DyLight800-N-hydroxysuccinimide ester for 1 hr on ice at a molar ratio of 1.5:1 (dye:protein) or 5:1 for higher labeling density. To quench free Alexa488-TFP or DyLight800-NHS, Tris-HCl pH 7.5 was added to a final concentration of 5mM. For the more densely-labeled substrate AlexaFluor 488 C_5 maleimide (Life Technologies) was added at a 10:1 (dye:protein), incubated for 2 hrs and quenched by addition of 1mM DTT. The densely-labeled substrate contained an average of 3 Alexa488-maleimide and 5 Alexa488-TFP molecules per protein.

All labeled CPY*TM variants were purified by size exclusion chromatography on a Superdex 200 16/600 column equilibrated with buffer B_F.

Reconstitution into proteoliposomes

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Avanti Polar Lipids was solubilized in chloroform. The solvent was removed under a N_2 stream followed by placing the sample into a SpeedVac for 3 hrs. DOPC was resuspended to 5mM in buffer R (50mM HEPES, pH 7.4, 150mM KCl, 2.5mM $MgCl_2$, 100μM TCEP) by vortexing. Lipids were completely solubilized in 2.2% (32mM) Triton X-100 (Anatrace) for 20 min at room temperature. CPY*-TM substrates were added to 2.5μM and the mixture was incubated on ice for 40 min. The detergent was removed by gradual addition of Bio-Beads SM2 (Bio-Rad).

For color switching assays, 10mM $CaCl_2$ and 50μM GGGC-coupled-fluorophore were added to the proteoliposomes containing CPY*-TM. After incubation for 10 min on ice, 2μM sortase was added and the reaction was incubated on ice for 2 hrs. No further increase in labeling was observed when the reaction time was extended to 16 hrs. The proteoliposomes were mixed 1:1 with 80% glycerol in buffer R. The proteoliposomes were overlaid with 40% glycerol, 30% glycerol, 15% glycerol and 0% glycerol (all prepared in buffer R). The step gradient was centrifuged for 3 hrs at 50,000 rpm in a TLS-55 rotor (RCF_{avg} 166,180). Six fractions were collected, starting from the top of the gradient. Proteoliposomes formed a sharp band between the 15% and 0% glycerol layers. Sortase and GGGC-fluorophore stayed in the 40% glycerol layer.

To incorporate Hrd1 into the proteoliposomes, the floated samples were partially solubilized by addition of 0.12% DMNG (1.3mM) on ice for 30 min. Hrd1 was added to 2μM and the mixture incubated on ice for 60 min. To remove DMNG, the samples were applied to three successive detergent removal columns (Pierce), each time for 30 min. The bed volume of the detergent removal columns was at least 4 x the sample volume. The proteoliposomes were then mixed 1:1 with 80% glycerol in buffer R and subjected to glycerol-step gradient centrifugation, as described above.

Determination of protein orientation in proteoliposomes

To test the orientation of Hrd1, proteoliposomes were incubated with 20μM TEV protease for 2 hrs at 4°C in the absence or presence of 1% Triton X-100.

To test the accessibility of the cysteines in reconstituted substrate, methoxypolyethylene glycol maleimide (Mal-PEG_{5000}, Sigma) was added on ice at different concentrations for 30 min. The reaction was stopped by adding 2mM DTT for an additional 5 min on ice, followed by 5x SDS-sample buffer. To test the accessibility of the lysines in reconstituted substrate, proteoliposomes were incubated with Rsp5 as described below. As a second test for the orientation, proteoliposomes were incubated with rLys-C protease (Promega) at the indicated concentrations at 25°C for 60 min.

Ubiquitination assays

Ubiquitination assays were performed at 30°C in buffer U_D (50mM HEPES, pH 7.4, 150mM KCl, 2.5mM $MgCl_2$, 50µM TCEP, 120µM DMNG). The concentrations of the protein components were: 0.2µM Uba1, 2µM Ubc7, 2µM Cue1ΔTM, 0.2µM Hrd1, 0.2µM substrate, 100µM ubiquitin, 0.6µM BSA. 2mM ATP was added to start the reaction. The samples were analyzed by SDS-PAGE and scanning with an Odyssey scanner at 700nm and 800nm (Li-Cor). Unless otherwise noted, yeast ubiquitin was used (Boston Biochem). Ubiquitin K48R were variants of human ubiquitin (Boston Biochem). Pre-formed di- and tetra-ubiquitin chains were terminated with K48R and are derived from human ubiquitin (LifeSensors).

Rsp5 ubiquitination was performed at 30°C in buffer U (without DMNG). The concentrations of the protein components were: 0.2µM Uba1, 2µM Ubc1, 2µM Rsp5, 100µM ubiquitin, 0.6µM BSA. 2mM ATP was added to start the reaction.

For ubiquitination reactions with proteoliposomes, the vesicles made up 3/5 of the total volume. Each of the components were added (except ATP) and the proteoliposomes were brought to 30°C for 5 min prior to adding ATP.

Proteoliposome pull-down experiments

To pull on Hrd1 in reconstituted proteoliposomes, the vesicles in buffer U_B (without DMNG, +0.6µM BSA) were incubated with 4µg/µL streptavidin magnetic beads (Pierce) for 1 hr at 4°C. The beads were washed three times with buffer U_B (without DMNG, +0.6µM BSA). The vesicles were eluted with buffer U +2mM biotin and analyzed by SDS-PAGE.

To pull on the substrate molecules, proteoliposomes were generated with CPY*-TM^{800}. The color of substrate molecules with an accessible C-terminus was switched by incubation with sortase and an Alexa488-labeled peptide. $Hrd1^{680}$ was reconstituted into these proteoliposomes and anti-Alexa Fluor 488 antibodies (Molecular Probes) were added to 0.4µg/µL. After incubation for 30 min at 4°C, magnetic protein G beads (Millipore) were added to 5µg/µL and the incubation continued at 4°C for 30 min. The beads were washed three times with buffer U_B (without DMNG, +0.6µM BSA). The bound and unbound vesicles were analyzed by SDS-PAGE.

Retrotranslocation assays using fluorescence quenching

Proteoliposomes were prepared as described above, with the exception that the CPY*-TM was also labeled on free amines (1.5 dye molecules per protein) with Alexa488-TFP prior to reconstitution. All lysines are within the CPY* domain. In addition, after reconstituting Hrd1, anti-Alexa488 antibodies were added before the final flotation. Each of the ubiquitination components were added (except ATP) and the proteoliposomes were brought to 30°C for 5 min prior to adding ATP. The samples were placed into a SpectraMax M5 plate reader (Molecular Devices) set to record measurements every 5 min (Excitation at 493nm / Emission at 518nm). Time "0" was recorded, then ATP was added along with a fresh aliquot of anti-Alexa488 antibody or Triton X-100 (each where indicated). After 60 min, all samples received 0.5% Triton X-100 to completely solubilize the proteoliposomes and completely quench Alexa488 fluorescence where antibody was re-added after time "0".

Hrd1-substrate binding assays

Binding assays were performed at room temperature in buffer U_D (50mM HEPES, pH 7.4, 150mM KCl, 2.5mM $MgCl_2$, 50µM TCEP, 0.1µM BSA, 120µM DMNG). Increasing concentrations of Hrd1-SBP were immobilized on 20µL pre-washed magnetic streptavidin beads (Pierce) for 30 min. The beads were washed three times with buffer U_D. Substrate was added to 0.1µM and the incubation continued for 1 hr. The beads were washed three times with buffer U_D and the proteins were eluted with buffer U_D supplemented with 2mM biotin. Bound and unbound material was analyzed by SDS-PAGE and scanning with an Odyssey scanner (Li-Cor). Quantification of both bound and unbound material was used to determine the percentage of bound substrate.

To test the affinity of substrate for ubiquitinated Hrd1, Hrd1-SBP was incubated with streptavidin beads for 30 min. All of the required ubiquitination components were added as described above, together with either wild-type ubiquitin, ubiquitin (K48R), or di- or tetra-ubiquitin containing ubiquitin (K48R) as a chain terminator. The samples were incubated for 1 hr at 30°C, and the beads were washed three times with buffer U_D. Substrate was added as before, and after incubation with the substrate, the beads were washed three times with buffer U_D. The proteins were eluted with buffer U_D supplemented with 2mM biotin and analyzed by SDS-PAGE.

Cycloheximide-chase experiments

Cycloheximide-chase degradation assays were performed as described (Gardner et al., 1998) with the following modifications. Yeast cells were grown to mid-log phase (0.4-0.7 OD_{600}/mL) in 50mL synthetic dropout media. The cells were pelleted at 2,000 x g for 5 min and resuspended to 2.5 OD_{600}/mL in fresh medium supplemented with 50µg/mL cycloheximide. A 2mL aliquot was taken as the "0 min" timepoint and pelleted. The cell pellet was either flash frozen in liquid N_2 or resuspended in lysis buffer. The remaining culture was incubated at 30°C with samples taken at 30 min, 60 min, and 90 min.

Cell pellets were incubated with lysis buffer (10mM MOPS, pH 6.8, 1% SDS, 8M urea, 10mM EDTA, protease inhibitors) at 25 OD_{600}/mL with an equivalent volume of acid-washed glass beads (0.1mm, Bio-Spec). After vortexing for 2 min, an equal volume of urea sample buffer (125mM Tris pH 6.8, 4% SDS, 8M urea, 10% β-mercaptoethanol) was added. The samples were incubated at 65°C for 5 min before SDS-PAGE and western blotting with an HA-antibody (clone 3F10, Roche).

Detection of Hrd1 ubiquitination *in vivo*

A centromeric plasmid encoding a Hrd1-His_{10} under the endogenous *HRD1* promoter was transformed into *hrd1Δ* cells. The cells were grown to mid-log phase and lysed in 50mM HEPES pH 7.4, 300mM KCl, protease inhibitors, 1mM PMSF, and 5mM NEM (to inhibit deubiquitinating enzymes). After centrifugation at 2,000 x g for 10 min, and the supernatant was centrifuged for 30 min in a Ti45 rotor at 42,000 rpm (RCF_{avg} 138,001). The membranes were solubilized in buffer S_{UXI} (buffer S+ 6M urea, 1.5% Triton X-100 final and 25mM imidazole) for 1 hr at 4°C. His-tag Dynabeads were added (0.25 mL per 1,500 OD cells) and the incubation continued at 4°C for 1 hr. The beads were washed 3 times with buffer S_{UXI} (buffer S+ 6M urea, 0.5% Triton X-100 final, 25mM imidazole). Hrd1-His_{10} was eluted with buffer S_{UX} (+400mM imidazole). The samples were analyzed by SDS-PAGE and western blotting with Hrd1- and ubiquitin- antibodies (clone P4D1, Santa Cruz).

Table S1. Multi-spanning ubiquitin ligases of the ER, Related to Figure 7.

Ligase	**Number of TM segments**	**Function**	**Reference**
S. cerevisiae			
Hrd1	6	ERAD-L, -M	(Carvalho et al., 2006; Vashist and Ng, 2004)
Doa10	14	ERAD-C	(Carvalho et al., 2006; Kreft et al., 2006; Swanson et al., 2001; Vashist and Ng, 2004)
Asi1-3	12	Inner nuclear membrane protein degradation	(Foresti et al., 2014; Khmelinskii et al., 2014)
Mammals			
Hrd1	6	ERAD-L, -M	(Bernasconi et al., 2010; Kikkert et al., 2004)
gp78	6	ERAD-M	(Fang et al., 2001; Song et al., 2005)
Teb4	14	ERAD-C	(Hassink et al., 2005; Kreft et al., 2006)
Trc8	10	MHC class I degradation	(Stagg et al., 2009)
TMEM129	3	MHC class I degradation	(van de Weijer et al., 2014; van den Boomen et al., 2014)

Supplemental References

Bernasconi, R., Galli, C., Calanca, V., Nakajima, T., and Molinari, M. (2010). Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ERAD-LS substrates. J. Cell Biol. *188*, 223-235.

Chen, I., Dorr, B.M., and Liu, D.R. (2011). A general strategy for the evolution of bond-forming enzymes using yeast display. Proc. Natl. Acad. Sci. USA *108*, 11399-11404.

Fang, S., Ferrone, M., Yang, C., Jensen, J.P., Tiwari, S., and Weissman, A.M. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *98*, 14422-14427.

Frey, S., and Gorlich, D. (2014). A new set of highly efficient, tag-cleaving proteases for purifying recombinant proteins. J. Chrom. A *1337*, 95-105.

Gaynor, E.C., te Heesen, S., Graham, T.R., Aebi, M., and Emr, S.D. (1994). Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. J. Cell Biol. *127*, 653-665.

Gietz, R.D., and Schiestl, R.H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Prot. *2*, 31-34.

Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V., and Wiertz, E. (2004). Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J. Biol. Chem. *279*, 3525-3534.

Kreft, S.G., Wang, L., and Hochstrasser, M. (2006). Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). J. Biol. Chem. *281*, 4646-4653.

Song, B.L., Sever, N., and DeBose-Boyd, R.A. (2005). Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. Mol. Cell *19*, 829-840.

Stagg, H.R., Thomas, M., van den Boomen, D., Wiertz, E.J., Drabkin, H.A., Gemmill, R.M., and Lehner, P.J. (2009). The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. J. Cell Biol. *186*, 685-692.

Swanson, R., Locher, M., and Hochstrasser, M. (2001). A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. *15*, 2660-2674.

van de Weijer, M.L., Bassik, M.C., Luteijn, R.D., Voorburg, C.M., Lohuis, M.A., Kremmer, E., Hoeben, R.C., LeProust, E.M., Chen, S., Hoelen, H., *et al.* (2014). A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. Nat. Comm. *5*, 3832.

van den Boomen, D.J., Timms, R.T., Grice, G.L., Stagg, H.R., Skodt, K., Dougan, G., Nathan, J.A., and Lehner, P.J. (2014). TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I. Proc. Natl. Acad. Sci. USA *111*, 11425-11430.