Supporting Information

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SI Materials and Methods

Antibodies and Reagents. Antibodies against GFP were from Invitrogen (3E6 and rabbit polyclonal anti-GFP), Clontech (JL-8), and Rockland Immunochemicals (anti-GFP-biotin). Other antibodies used were against Hrd1 (Abgent), Derlin-1 (Abcam), vimentin (Sigma), ubiquitin (EMD Millipore), and Thy1.1 (eBioscience). HRP-conjugated secondary antibodies and streptavidin were from Jackson Immunoresearch and Invitrogen, respectively. The CLIP-reactive CerCLIP.1 antibody has been previously described (1, 2). Inhibitors were from Sigma (BFA), Enzo Life Sciences (epoxomicin, MG-132), and Roche (zVAD-fmk).

Plasmids. Plasmids encoding ZV1, ZV2, V1Z, and V2Z in pcDNA3.1 were a kind gift from Stephen Michnick (University of Montreal, Montreal, QC, Canada). The N-terminal half of Venus (V1) includes amino acids 1–157 and the C-terminal half (V2) includes amino acids 158–238. Amino acid numbering of Venus began with the valine in position 2 according to previous publications (3). The H2-K^b signal sequence was added to these coding sequences by PCR and inserted into pcDNA or MSCV-Thy1.1 by standard molecular biology techniques. The addition of the CLIP epitope (LPKPPKPVSK) and glycosylation mutations were performed by Quikchange site-directed mutagenesis (Agilent Technologies). Single-molecule Venus was created by PCR of each Venus half and subsequent overlapping PCR and was inserted into pcDNA3.1 and MSCV-Thy1.1. Correct coding regions were all verified by sequencing. A1AT-NHK-eGFP was a kind gift from Ron Kopito (Stanford University, Stanford, CA). pcDNA3.1 encoding DN Hrd1 has been described previously (4) and was kindly provided by Paul Lehner (University of Cambridge, Cambridge, United Kingdom).

Transfections, Inhibitor Treatment, and FACS Analysis. HEK293T cells were transfected in 96-, 12-, or 6-well plates or 10-cm dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. In most experiments, MSCV-Thy1.1 was cotransfected with split Venus or Venus vectors to mark transfected cells. In such cases, split Venus halves were cotransfected with MSCV-Thy1.1 at a ratio of 1:1:0.5. The same ratio was used when cells were cotransfected with Venus, DN Hrd1, and MSCV-Thy1.1. A 3:1 ratio was used when cells were cotransfected with Venus and MSCV-Thy1.1. After 18–24 h of transfection, epoxomicin (0.5–1 μM), MG-132 (8 μM), zVAD-fmk (20 μM), or BFA (5–10 μg/mL) were added for 6 h at 37 °C. Cells were harvested with trypsin, stained with anti-Thy1.1-APC for 30 min, washed, and fixed. For most FACS experiments, cells were transfected in 96-well plates, and triplicate wells were pooled and analyzed on a FACSCalibur, LSRII, or Accuri flow cytometer (BD). In experiments containing Thy1.1 expression, live cells were gated and then gated on Thy1.1 expression. Typical transfection efficiencies ranged from 15 to 60%.

Immunoprecipitation and Western Blot. After washing in ice-cold PBS or TBS, cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) or 1% TX-100 (50 mM Tris, 150 mM NaCl) containing protease inhibitors (EDTA-Free Complete tablets; Roche) for 30 min on ice. The detergent soluble fraction was collected after centrifugation at 14,000 *g* for 10 min. Antibodies and protein A- or G-Sepharose beads were added, rotated at 4 °C for 60 min or overnight, and washed. Immunoprecipitates or cell lysates were electrophoretically separated on a 10–20% gradient (S1; Invitrogen) or 12% acrylamide gels (all others), transferred to an Immobilon-P membrane, blocked for 30 min in 5% nonfat dry milk, incubated with primary antibodies for 60 min, washed, and then incubated with HRP-conjugated secondaries or streptavidin. After washing, blots were developed with ECL substrate (Pierce) and exposed to film.

Microscopy. Cells were transfected in 6-well plates. After 4–6 h, DNA complexes were removed and cells were seeded in 24-well plates containing poly-L-lysine–coated coverslips and incubated overnight. Inhibitors were added for 6 h, cells were fixed with 4% (wt/vol) paraformaldehyde and mounted on slides using Prolong Gold Antifade (Invitrogen). In immunostaining experiments, cells were permeabilized with 0.1% TX-100 in PBS, blocked for 30 min, stained with primary antibodies for 60 min, washed, stained with Alexa-647 conjugated secondaries (Invitrogen) for 60 min, and mounted as above. Cells were analyzed on a Leica SP2 confocal microscope at 63× magnification.

Statistical Analysis. The *P* values were determined using Student's *t* test from at least three independent experiments. All error bars shown on graphs represent the SE. In FACS experiments in which cells were cotransfected with MSCV-Thy1.1, the geometric mean fluorescence intensity (GMFI) of untreated $Thy1.1^+$ cells was subtracted from treated cells. These values were used to determine the percent inhibition by DN Hrd1 and zVAD-fmk. In experiments in which MSCV-Thy1.1 was not cotransfected, the integrated GMFI was used. To determine the integrated GMFI, the percent $Venus^+$ cells was multiplied by the GMFI of the positive cells.

1. Denzin LK, Cresswell P (1995) HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell* 82(1):155–165.
2. Denzin LK, Robbins NF, Carboy-Newcomb C, Cresswell P (1994) Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* 1(7):595–606.
3. Nagai T, et al. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 20(1):87–90.
4. Kikkert M, et al. (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem* 279(5):3525–3534.

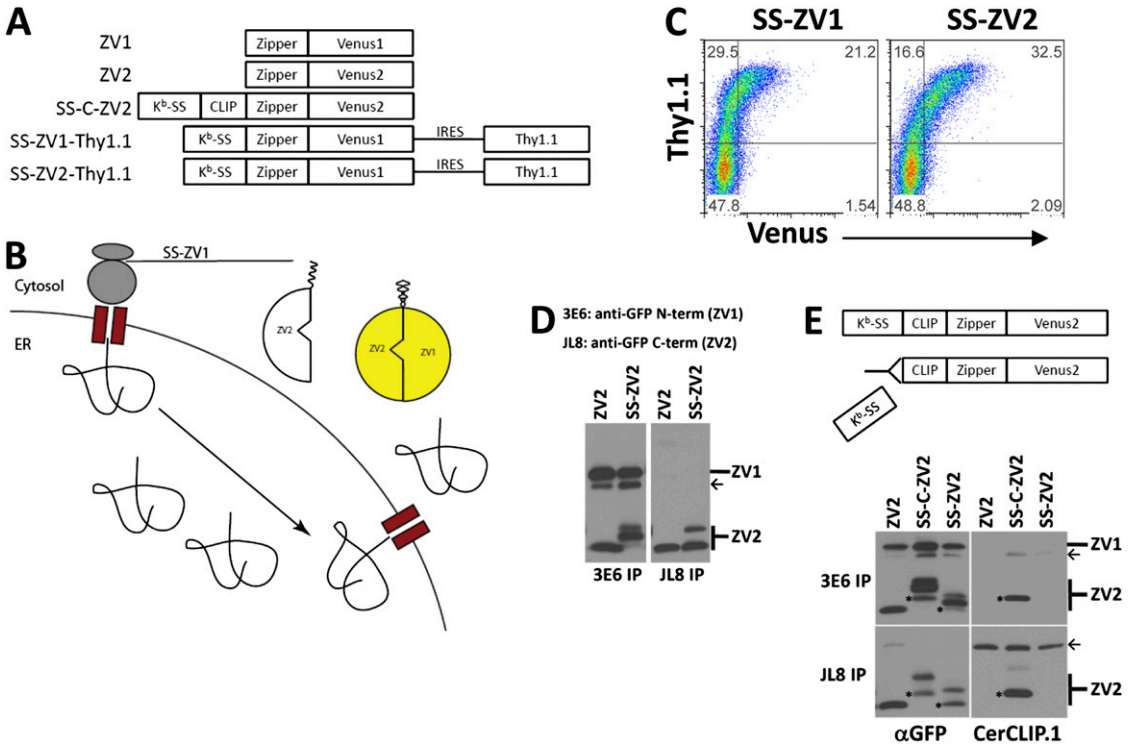

Fig. S1. Fluorescence arising from split Venus is from non-signal-sequence–cleaved protein. (*A*) Vectors used. These encode split Venus fused to a leucine zipper at either the *N* or C terminus. (*B*) Schematic depicting the split Venus ERAD system. See text for details. (*C*) FACS analysis of HEK293T cells transfected with MSCV-SS-ZV1-Thy1.1 or SS-ZV2-Thy1.1 and the corresponding half of Venus in the cytosol. (*D* and *E*) Cells transfected with ZV1 and the indicated ZV2 vectors were lysed in detergent and Venus immunoprecipitated using the anti-GFP antibodies 3E6 or JL-8. Note that 3E6 binds to the N-terminal segment of split Venus (ZV1) and will coprecipitate associated C-terminal segment (ZV2) but JL-8 only immunoprecipitates free C-terminal segment (ZV2). After separation by SDS/PAGE on a 10–20% gradient gel and transfer to Immobilon-P, membranes were blotted with anti-GFP or the CerCLIP.1 mAb, the latter of which only binds proteins with a free N terminus (i.e., only binds when signal peptide is removed). After 3E6 IP, which will IP any ER-targeted ZV2 that is associated with cytosolic ZV1, only a minor fraction has the signal sequence completely cleaved, and the major species in intermediate in size between cleaved and uncleaved. Blotting with CerCLIP.1 demonstrates that the slower migration of the intermediate band is not due to posttranslational modification of signal-sequence–cleaved protein, as only the fastest migrating band is reactive with CerCLIP.1. Asterisks denote the population that has the signal sequence completely cleaved. Arrows denote nonspecific bands.

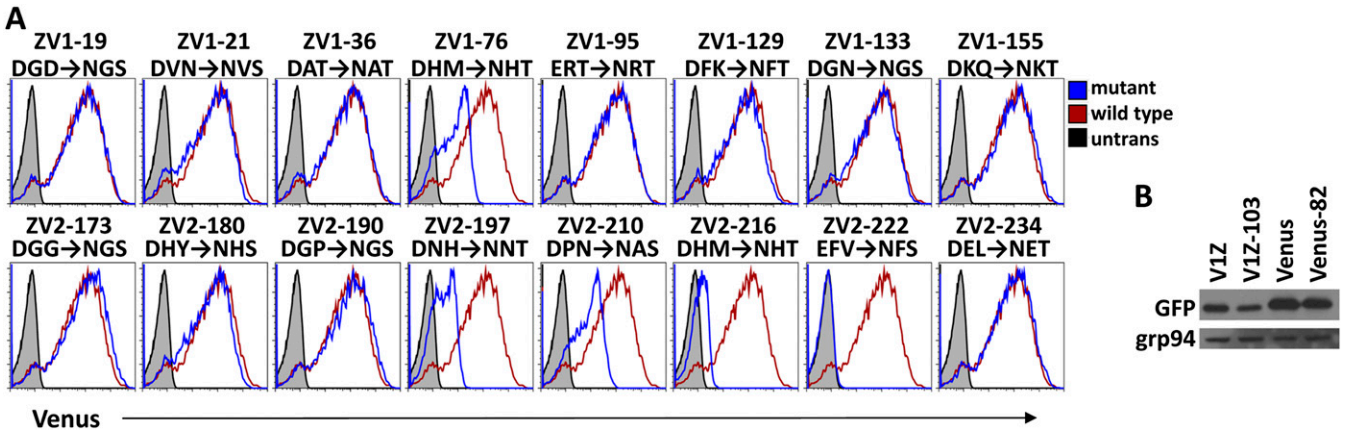

Fig. S2. Identification of deglycosylation-dependent split Venus mutants. (*A*) HEK293T cells were cotransfected with cytosolic wild type or the indicated mutant split Venus vector, the corresponding Venus half, and MSCV-Thy1.1. Histograms show Thy1.1+ cells. (*B*) Cells expressing the indicated constructs were lysed in RIPA buffer and detergent-soluble lysates analyzed for expression of Venus and grp94 by reducing SDS-PAGE/western blot.

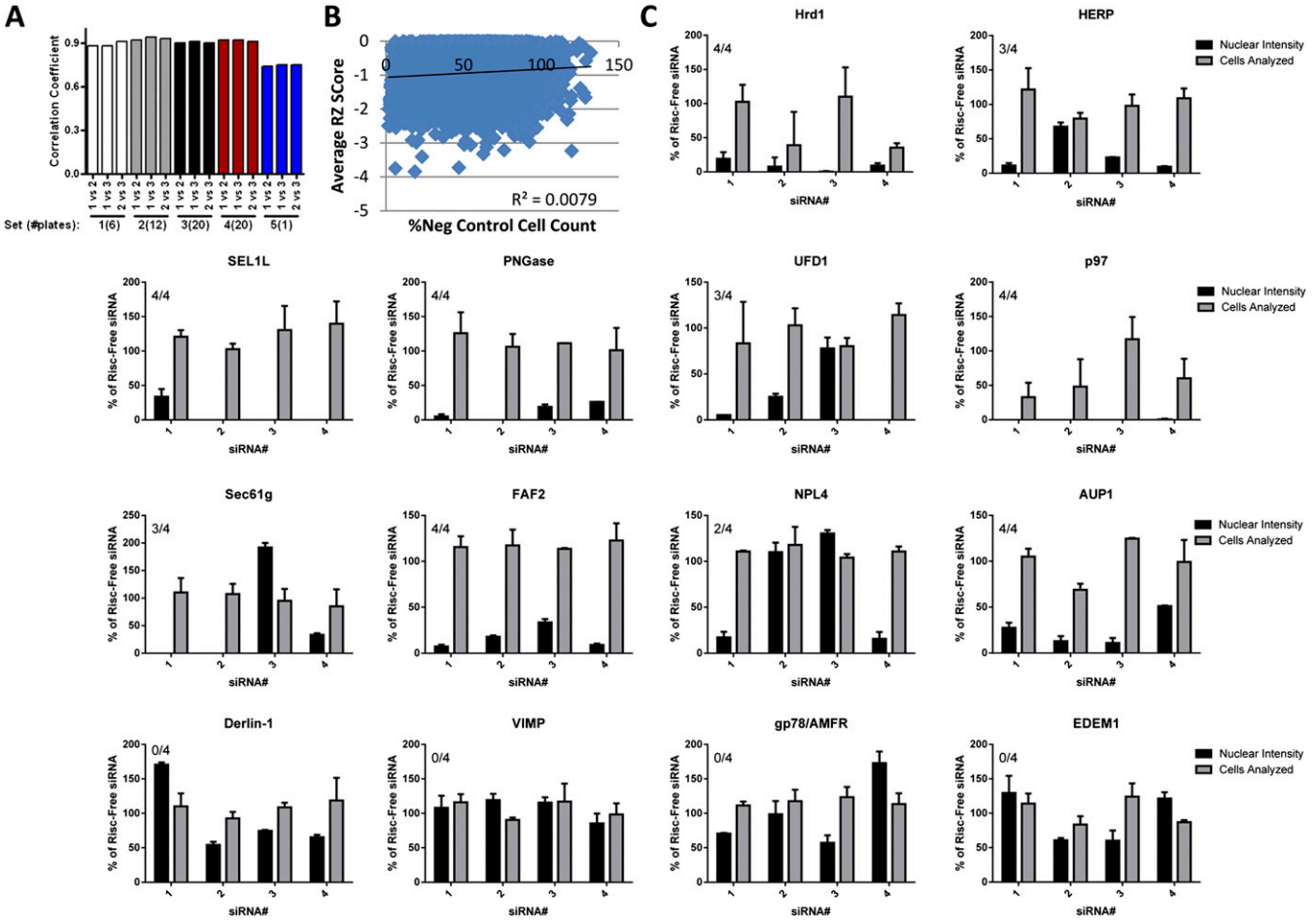

Fig. S3. Genome-wide siRNA screen performance and confirmation. (*A*) RZ scores were determined for each dataset and plotted versus replicate samples to determine the correlation coefficients. (*B*) The average RZ score and average percent cell count of negative control siRNA were plotted against each other to determine the relationship between fluorescence and number of cells/well. (*C*) The 293T.FluERAD cells were transfected with four individual siRNA per gene for 66–72 h, treated with 8 μM MG-132 for 6 h, and nuclear intensity measured by microscopy. Numbers on graphs denote the number of siRNAs that inhibit fluorescence at least 50% as well as the positive control PNGase siRNA pool.