Supplementary Methods

DSS1-3×FLAG cells. For recombinant expression and purification of human 26S proteasome, a HEK293F cell line stably expressing DSS1-3XFLAG was derived. To this end, a plasmid encoding DSS1/Rpn15 TEV-3XFLAG was constructed by conventional methods using the p3xFLAG-CMV14 construct (Sigma, St. Louis, MO), linearized by XmnI digestion and transfected into HEK293F growing adherently. Stable clones were isolated and candidate subclones were screened for FLAG reactivity by Western blotting and by LLVY-AMC proteolytic activity after FLAG immunoprecipitation.

For the initial validation of this approach as presented in the Supplemental figure 1 and Supplemental table 1 cells were grown in suspension and proteasomes were purified in presence of 100 mM NaCl (details of the purification described in the supplement). For all other experiments cells were cultured adherently in DMEM (Gibco) with 10% FBS (Sigma) + 100 μg/ml Geneticin (Gibco). Cells were harvested by trypsinization, washed twice with PBS and processed immediately or cell pellets were stored at -80°C.

Cell culture for quantitative proteomics. For SILAC experiments (Ong et al, 2002) cells were grown for at least 9 passages in heavy or light SILAC medium: for 1 L medium use 13 g DMEM powder (Gibco formula), 50 mg L-leucine, 0-15 mg L-proline, 3.7 g NaHCO3, 3.12 g HEPES, adjust pH to 7.2) supplemented with 10% dialyzed FBS (Hyclone) and 50 mg/ml L-Lysine and L-Arginine (light medium) or 50 mg/ml 13C6;15N2-L-Lysine and 13C6;15N4-L-Arginine (heavy medium) (Cambridge Isotope Laboratories, Inc.).

Proteasome purification. Cells were lysed by sonication in purification buffer (25 mM HEPES, pH7.4-KOH, 10% glycerol, 10 mM MgCl2, 1 mM ATP, 1x phosphatase inhibitor cocktail II (Sigma), 1 x Complete Mini protease inhibitor cocktail EDTA-free (Roche). The homogenate was cleared from debris by centrifugation (15 min 1500 g) followed by removal of the microsomal fraction (1 h 100,000 g). The clear supernatant was incubated with Anti-FLAG resin (Sigma) according to manufacturers instructions and eluted with 150 μg/ml 3xFLAG peptide (Sigma) The FLAG peptide was removed by dialysis (10 MWCO Mini-slide-A-Lyzer, Pierce) against the storage buffer (10 mM Tris pH 7.6-HCl, 25 mM KCl, 10 mM NaCl, 2.1 mM MgCl2, 1 mM DTT, 2 mM ATP, 25% glycerol, 0.1 mM EDTA pH 8). Proteasomes were stored at -80°C. For purifications where ubiquitin conjugates were removed, the proteasomes bound to the FLAG–resin were incubated for 1 h 4°C with or without 0.5 μM USP2 in one bed volume purification buffer. Then the resin was washed with another 20 bed volumes of purification buffer and proteasomes were eluted as described in the protocol.

Recombinant USP2. The recombinant Usp2 catalytic core used in this study was cloned and purified similarly to what has been described before (Renatus et al, 2006). The constructs were either N- or C-terminally His-tagged version of amino acids 260-605 of human USP2.

Proteomic Analyses. Purified 26S proteasome preparations were mixed in equal amounts. The mixed samples were fractionated on a 4-12% SDS-PAGE gel. The gel was sliced into 10 or 20 sections and tryptic in-gel digestion of the gel slices was performed.
Dried protein digests were reconstituted in a solution containing 1% formic acid, 2% acetonitrile and 97% water (v/v) for LC/MS/MS analysis.

Reconstituted protein digests were analyzed on a LC/MS/MS system comprised of an EASY-nLC (Proxeon, Thermo Fisher Scientific, West Palm Beach, FL), and a LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A 15 cm PicoFrit column (75 μm inner diameter) packed with ProteoPep II C18 packing material (New Objective, Woburn, MA) was used for online peptide separation. A linear gradient of 2-40% B (89.9% acetonitrile and 0.1% formic acid with A as 0.1% formic acid) was used to elute peptides over 85 min. Eluted peptides were sprayed into the mass spectrometer through a PicoView nanospray ion source (New Objective, Woburn, MA). MS1 scans were acquired using an AGC target of 1x10^6 for the FT analyzer, and a resolution of 100,000 at 400 amu. Each MS1 scans was followed by 10 data-dependent CID MS/MS scans in the iontrap.

Raw MS data were processed using a combination of MaxQuant (version 1.0.13.13) (Cox & Mann, 2008) and Mascot (version 2.2.03, Matrix Science, London, UK). MaxQuant was used for post-acquisition precursor m/z calibration, MS/MS spectrum peak picking, SILAC peptide ratio calculation, as well as protein grouping and quantification. Database searching was done by using Mascot. For peptide/protein identification, the following modifications were included: carbamidomethylation (Cys, fixed), oxidization (Met, variable), N-acetylation (protein, variable), and pyro (Gln, variable). Trypsin specificity was set to exclude cleavages between Lys-Pro and Arg-Pro. Up to two missed cleavages and three labeled SILAC residues (Lys and Arg) per peptide were allowed. The MS/MS data were searched against a catenated database combining the IPI human database version 3.63 (84,118 sequences), the reversed sequences of all sequences in the IPI human database, and 262 commonly observed contaminants. The global false discovery rate for both peptides and proteins were set to 0.01. The posterior error probability threshold for peptide identification was set to 1 (no filtering). Minimum peptide length was 6 residues. Mass tolerances for precursor ions and product ions were 7 ppm (after calibration by MaxQuant) and 0.5 Da, respectively. For protein quantitation, three modifications were included, carbamidomethylation (Cys), oxidization (Met), and pyro (Gln), along with all unmodified peptides. Requantify function was enabled to capture SILAC pairs that were missed in the initial SILAC pair identification.

GG-peptide analysis: Peptides with di-glycine modified to lysines were enriched following protocols and analysis described by Kim et.al. (Kim et al, 2011). In short purified proteasomes digested with trypsin were enriched using K-GG motif antibody. The K-GG enriched peptides were separated by online nLC-MS on a Orbitrap Velos (Thermo).

Proteasome inhibition and wash out. 4 x 15 cm plates of cells (about 70% confluent) were treated with either 0.01 % DMSO, 10 µM MG132, 1 µM Bortezomib, or 400 nM Epoxomycin. After 4 h the inhibitor was removed by washing twice with cell culture medium. One plate was harvested by trypsinization at each 0, 1, 4 and 20 h after removal of the inhibitor and cell pellets stored at -80°C until further analysis. Cell pellets were lysed by sonication in a buffer containing 10% (v/v) Glycerol, 25 mM Heps pH 7.4, 5 mM MgCl₂, 1 mM ATP, 1 mM NEM, 1 mM Orthophenantroline, 1 x Phosphatase Inhibitor Mix (Sigma-Aldrich), 1 x Complete Protease Inhibitor Cocktail (Roche). Cell
and debris was removed by a 30 minute spin at 20,000 g. Proteasomal peptidase activity was determined as described previously (Besche et al, 2009) using Suc-LLVY-amc (Bachem) and free 7-Amino-4-methylcoumarin (amc; Bachem) as standard curve. Western blot analysis was performed according to standard chemiluminescence protocols.


Fang NN, Ng AH, Measday V, Mayor T (2011) Hul5 HECT ubiquitin ligase plays a major role in the ubiquitylation and turnover of cytosolic misfolded proteins. *Nature cell biology* **13**: 1344-1352


