Supplemental Materials

Molecular Biology of the Cell

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Supplemental Information

Supplemental Materials and Methods include plasmids, antibodies, proteins, size-exclusion spin column assay, native-PAGE, fluorogenic peptidase assay, glycerol gradient sedimentation assay, immunoblotting assay and nuclear and cytoplasmic fractionation. There are also five supplemental figures and figure legends.

Supplemental Information

Supplemental Materials and Methods

Plasmids The following plasmids were subcloned into appropriate constructs for mammalian or bacterial expression, including pRK7-HA-Ubiquitin, pRK7-Myc-Ubiquitin, pRK7-HA-UbE2D1, pRK7-HA-UbE2D1(C85A), pRK7-Myc-UbE3A, pRK7-Myc-UbE3A(C843A), pRK7-FLAG-TRIM33, pGEX6p-1-UbE3A and pET28(a)-Ubiquitin. All plasmid sequences were confirmed by the Sequencing Core Facility at University Colorado School of Medicine. pCMV-Myc-UbE3C and pCMV-Myc-UbE3C(C1051A) plasmids were gifts from Dr. Robert E. Cohen at the Colorado State University.

Antibodies The following antibodies were purchased: Rpn1, Rpn2 and UbcH10 (Boston Biochem); beta-actin and FLAG-M2 (Sigma); Myc (Santa Cruz); HA (Covance); UbE3C, Rpn5, Rpn6, Rpt4, Adrm1, GPADH and Rpt6 (GeneTex); Ecm29 and Uch37 (Epitomics); Adrm1 and Herc2 (Bethyl); Rpn11 (Zymed); UbE1 and E6AP (Enzo Life Sciences). The beta-5, Usp14, Rpt1, Rpt3, Rpt5, Rpn12 and S5a antibodies were gifts from Dr. George N. DeMartino at the University of Texas Southwestern Medical Center.

Proteins Human ubiquitin activating enzyme E1 and an E2 screening kit were purchased from UBPBio. GST-UbE3A was purified from *E. coli BL21(DE3) RIPL* cells (Agilent Technologies). Cells harboring the pGEX6p-1-UbE3A plasmid were grown at 37 ^{0}C until OD_{600} = 0.4. Cells were cooled down to 16 ^{0}C before the addition of 0.05 mM IPTG for induction of 16 hrs. Cell pellets from a 2 L culture were resuspended into 40 mL lysis buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 2 mM βME, 1X Leupeptin, 1X PMSF), followed by sonication and centrifugation at 30,000 x*g* for 30 min at 4 ^{0}C. Proteins were purified from the supernatant using Glutathione resin according to the manufacturer's instruction. UbE3A was released from the resin using PreScission protease.

Size-exclusion spin column assay Binding of polyubiquitin chains on the 26S proteasome was assayed using a size-exclusion spin column assay similar to the method described previously [1]. 40 μL reactions containing 80 nM purified human 26S proteasome and 2.5 μM Ubal were incubated at 37 ^{o}C for 10 min. 1 μM K48-Ub_2, 500 nM K48-Ub_4 or 250 nM K48-Ub_8 was then added for an additional 10 min incubation. Afterward, reaction mixtures were loaded onto home-made Sephadex G100 spin columns, centrifuged at 1000 x*g* for 3.5 minutes and the flow-through was collected. Proteins in each flow-through were analyzed by immunoblotting. Similar assays were also used to determine binding of K48-Ub_4 on non-ubiquitinated or ubiquitinated 26S proteasome. Non-ubiquitinated 26S proteasome was prepared by incubating 80 nM purified 26S proteasome with 50 μM Ub and 2.5 μM Ubal for 1 hr; ubiquitinated 26S proteasome was prepared by incubating 80 nM purified 26S proteasome with 50 μM Ub, 2.5 μM Ubal and 2 μM UbE2D1 for 1 hr.

Native-PAGE and SUC-LLVY-AMC activity assay 4% native-PAGEs were made as described previously [2]. 3 μg or 1 μg purified 26S proteasome was loaded in each well for Coomassie staining or immunoblotting assays, respectively. In-gel overlay assays were performed as described previously by using SUC-LLVY-AMC (UBPBio) as the fluorogenic substrate to monitor the chymotrypsin-like activity of the proteasome. The released AMC fluorescence was visualized by an AlphaImager (Alpha Innotech).

***Glycerol gradient sedimentation* assay** For analytical glycerol gradient sedimentations, 1.8 mL of 12.5-40% glycerol gradients were prepared as described previously [3]. In these assays, 100 μL purified 26S proteasome (30 μg, ubiquitinated or non-ubiquitinated) was loaded onto a glycerol gradient and centrifuged using a Ti55 rotor for 3 hours at 55,000 rpm at 4 ^{0}C. 90 μL fractions were collected. 15 μL of each fraction was loaded onto SDS-PAGE gels for immunoblot analysis.

Immunoblotting assay Proteins separated on SDS-PAGEs were transferred to nitrocellulose membranes, which were then probed with appropriate primary antibodies in 3% BSA overnight at 4 ^{0}C. After washing with 1X TTBS for three 10 min washes, each membrane was incubated with a secondary antibody of anti-mouse or -rabbit immunoglobulin-horseradish peroxidase at 1:5,000-20,000 dilutions for 2 hr. The signal was developed with Pierce ECL Western Blotting Substrates and visualized using a ChemiDoc MP Imaging System (Bio-Rad).

Nuclear and cytoplasmic fractionation Pellets from two 15 cm plates of 293T Rpn11-HTBH cells were resuspended in 4 ml of the proteasome purification buffer. Cells were allowed to swell on ice for 10 minutes followed by homogenization with 10 strokes in a Dounce homogenizer. The mixture was spun for 4 minutes at 500 x*g*. The supernatant was removed and respun at 16,000 x*g* to remove any cell debris and the supernatant was collected as the cytoplasmic fraction. The pellet from the 500 x*g* spin was washed twice and resuspended in 2 ml proteasome purification buffer. The mixture was then sonicated with 4 pulses of 5 seconds on 20% power using a 550 Sonic Dismembrator (Fisher Scientific). The lysate was spun at 16,000 x*g* for 4 minutes and the supernatant was collected as the nuclear fraction.

References

1. Jacobson,A.D. *et al.* The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26S proteasome. *J. Biol. Chem.* **284**, 35485-35494 (2009).
2. Elsasser,S., Schmidt,M., & Finley,D. Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol.* **398**, 353-363 (2005).
3. Koulich,E., Li,X., & DeMartino,G.N. Relative structural and functional roles of multiple deubiquitylating proteins associated with mammalian 26S proteasome. *Mol. Biol. Cell* **19**, 1072-1082 (2008).

Supplemental Figure Legends

Figure S1. Characterization of purified human 26S proteasome

A. Coomassie stained SDS-PAGE of 3 μg 26S proteasome purified from 293T Rpn11-HTBH cells. Pulldown from cells expressing the HTBH tag was loaded as a control.
B. Coomassie stained native-PAGE of 3 μg purified human 26S proteasome (left gel) and in-gel overlay assay (right gel) using SUC-LLVY-AMC to monitor the chymotrypsin-like activity of the proteasome.

Figure S2. Ubiquitinated proteins in purified 26S proteasome run similarly as those of *in vitro* ubiquitinated ones on SDS-PAGE

26S proteasome was subjected to *in vitro* ubiquitination (lane 1, 1 μg) and loaded side-by-side with 26S proteasome that was purified using the method to preserve ubiquitination (lane 2, 1 μg) on SDS-PAGE and analyzed by immunoblotting.

Figure S3. Ubiquitinated Adrm1, S5a, Rpt5 and Uch37 associate with the 26S proteasome

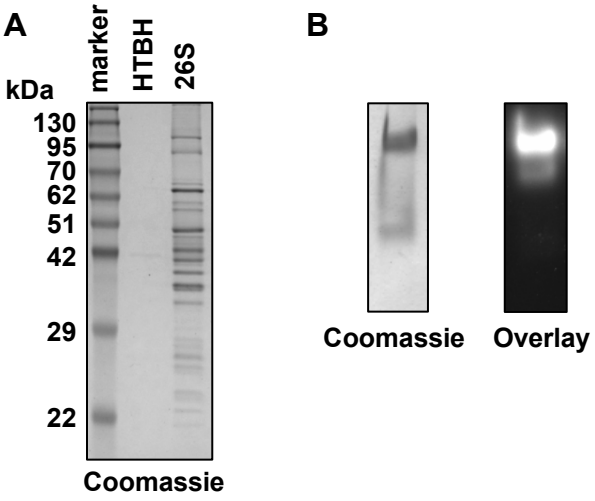
Non-ubiquitinated and ubiquitinated proteasome were obtained using reactions similar to those described in Figure 1C. 30 μg 26S proteasome was separated by 12.5-40% glycerol gradient sedimentations. Proteins in the glycerol gradient fractions were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Ubiquitinated species are marked with asterisks on the side of each blot. The band marked with an asterisk in the blot at fraction 7 for Adrm1 is likely a ubiquitinated protein cross-reacting with the anti-Adrm1 antibody.

Figure S4. The human 26S proteasome binds longer polyUb chains more efficiently than shorter chains

Immunoblot analysis of the flow-through from the size-exclusion spin column assay. K48-linked Ub_2, Ub_4 or Ub_8 was incubated with or without proteasome and applied to the spin columns for centrifugation. Only proteasome bound chains should pass through the spin columns in this assay. See Supplemental Materials and Methods for details.

Figure S5. Cellular stress affects ubiquitination of the 26S proteasome

A. Acute proteasome inhibition decreases Rpt5 ubiquitination. Proteasomes purified from untreated 293T Rpn11-HTBH cells or cells treated with 10 μM MG132 (two hours) were separated by SDS-PAGE followed by immunoblotting.
B. Long time proteasome inhibition increases proteasome ubiquitination. Proteasomes purified from untreated 293T Rpn11-HTBH cells or cells treated with 10 μM MG132 (16 hours) were separated by SDS-PAGE followed by immunoblotting.
C. Heat shock has no effect on proteasome ubiquitination. Proteasomes purified from 293T Rpn11-HTBH cells incubated at 37 ^{0}C or 42 ^{0}C (2 hours) were separated by SDS-PAGE followed by immunoblotting.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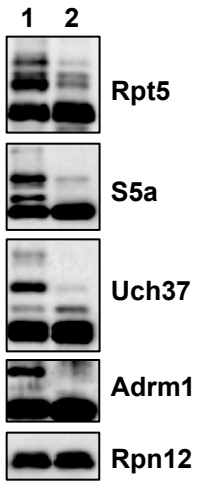
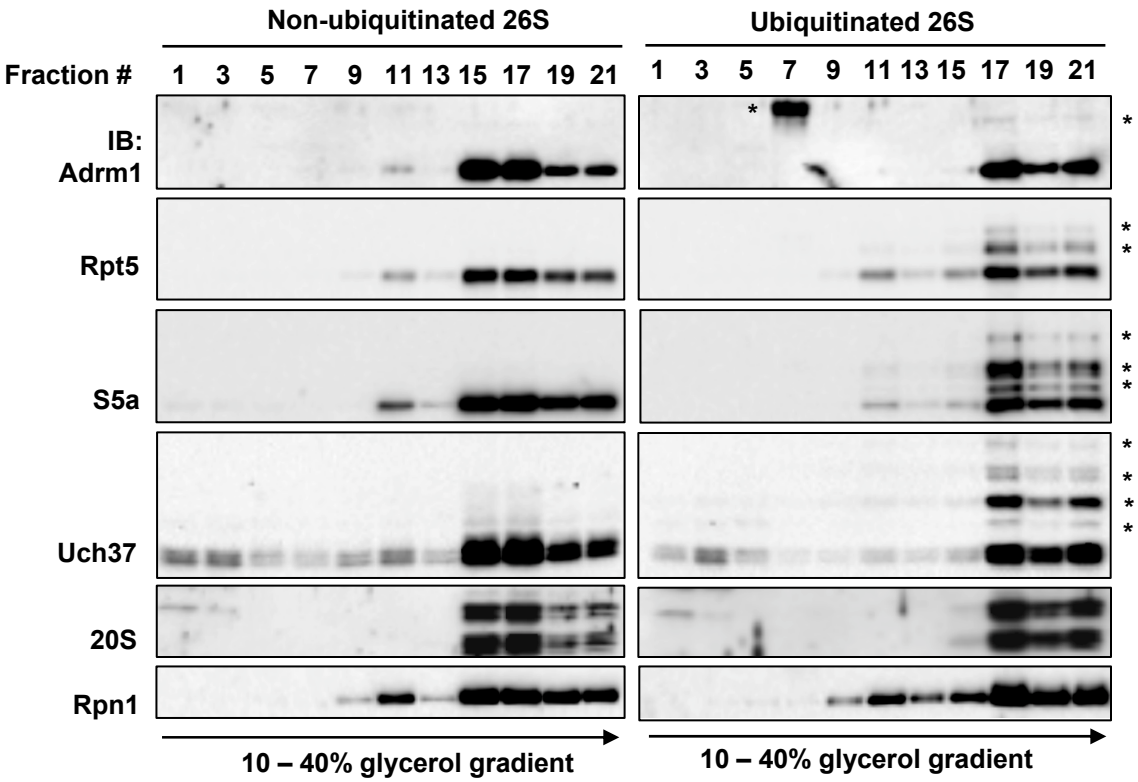
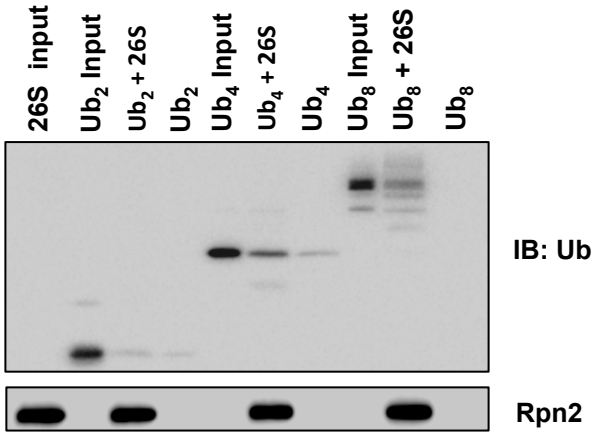
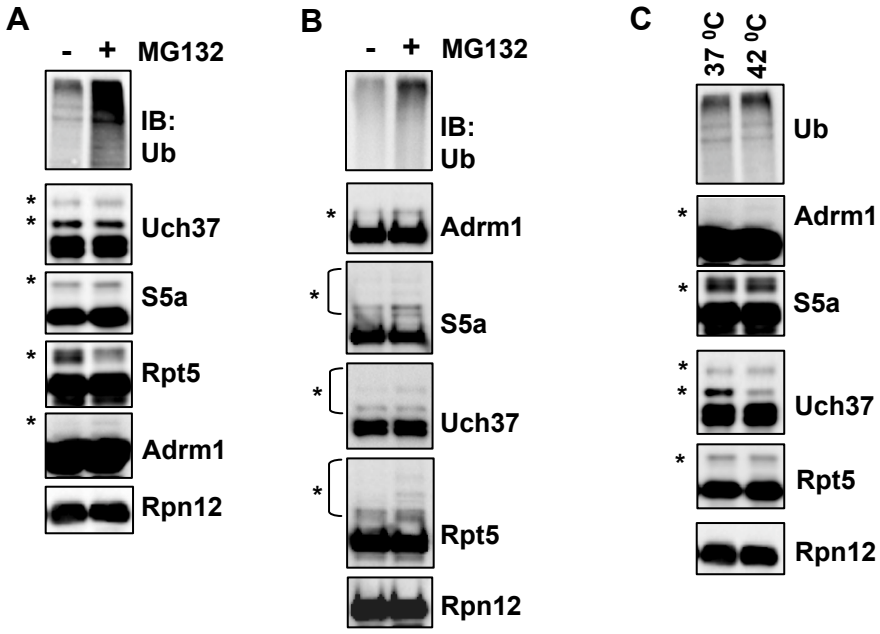


1. **In vitro ubiquitinated 26S proteasome**
2. **Rapidly purified 26S proteasome**



Liu_Supplemental Figure 3


26S input
Ub2 Input
Ub2 + 26S
Ub2
Ub4 Input
Ub4 + 26S
Ub4
Ub8 Input
Ub8 + 26S
Ub8
IB: Ub
Rpn2



Liu_Supplemental Figure 5