Supporting Information

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Materials. Materials for SDS/PAGE and Bradford reagent were from Bio-Rad. L-[^{35}S] methionine for in vitro translation and prestained molecular weight markers were obtained from GE Healthcare. ^{125}I for radiolabeling was from PerkinElmer. Tissue culture sera and media were from Biological Industries or Sigma. Mouse anti-HA (16B12) and mouse anti-Ub were from Covance, anti-Flag (M2) antibody was from Sigma, mouse anti-RGS·His was from Qiagen, and rabbit anti-p21 and anti-MyoD were from Santa Cruz. Anti-Mdm2 antibody (a mixture of 4B2 and 2A9 monoclonal antibodies) was kindly provided by Moshe Oren. Antibodies to the proteasome subunits were as follows: (*i*) mouse anti-human Rpt1 and rabbit anti-human α4 were from Biomol; (*ii*) mouse anti-α6 (ABR PAI-963 or monoclonal antibody 2–17 to human PSMA1) was kindly provided by Keiji Tanaka; and (*iii*) rabbit anti-S12 (PSMD7) was from Affinity BioReagents. Rabbit anti-UBB^{+1} was kindly provided by Fred van Leeuwen (1). Rabbit anti-Ub conjugates antibody have been described (2). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Protein G-Agarose and immobilized anti-HA were from Roche Molecular Biochemicals. Ni-NTA agarose was from Qiagen. Ub, DTT, ATP, adenosine 5′-[γ-thiotriphosphate] (ATPγS), 2-deoxyglucose, phosphocreatine, creatine phosphokinase, and Tris buffer (Trizma base) were from Sigma. Hexokinase was from Roche Molecular Biochemicals. Protease inhibitors mixture and *N*-carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG132) were from Calbiochem. Jet-PEI transfection reagent was from Polyplus Transfection. Ub aldehyde (Ubal) and *clasto*-Lactacystin were from Biomol. Reagents for ECL were from Pierce. TNT Wheat germ and *E. coli* T7 S30-based extract kits for coupled transcription-translation were from Promega. Suc-LLVY-AMC was from Bachem and Ub-AMC was from Biomol. Restriction enzymes were from New England Biolabs. All other reagents were of high analytical grade.

Methods. ***Cultured cells.*** HEK-293 and HeLa cells were grown at 37 °C in DMEM supplemented with 10% FCS and antibiotics (penicillin–streptomycin).

Mammalian cell transfection. Transient transfection of HEK-293 cells with expression vectors was carried out by using the jetPEI transfection reagent.

Plasmids and protein purification. All plasmids were constructed and manipulated by using standard molecular biological techniques. For cell expression, cDNAs were subcloned into pCS2 (Invitrogen; MyoD, c-MycHA, Mdm2, UBB^{+1}HA, UBB$^{+1/K29,48R}$, UBB^{+1}Y^{K29,48R}, UBB^{+1}HA^{K29,48R}), pIRES (Clontech; Flag-ODC and Flag-AZ), pcDNA3 (Invitrogen; UBB^{+1} and UBB$^{+1/K29,48R}$) or pCAGGS (3) (all other cDNAs coding for the different Ub^{VV} variants). cDNAs used for in vitro translation were subcloned into pCS2 (MyoD, Ring1B, XIAP-1) or pCDNA3.1 (Invitrogen; all other cDNAs coding for the different Ub^{VV} variants). For bacterial expression of Ub^{VV}His and its different variants and mutants (cloned into pET14b, Novagen), plasmids were expressed in BL21 (pLysS) *E. coli* and proteins were purified by using Ni^{2+}-NTA resin according to the manufacturer's instructions. Untagged Ub^{VV} (cloned in pET14b) was expressed and purified as described (4). Bacterially expressed MyoD was purified as described (5).

Protein concentration. Protein concentration was measured by the method of Bradford (6) using BSA as standard.

Preparation and fractionation of crude reticulocyte lysate. Reticulocytes were induced in rabbits and lysates were prepared and fractionated over DEAE cellulose onto fraction I (that contains Ub and several E2s) and fraction II (that contains the remaining enzymes of the UPS, but lacks endogenous Ub) as described (7).

Monitoring proteasomal DUB activity. Proteasome-associated DUB activity was measured by using Ub-AMC as a substrate according to the manufacturer's instructions. Briefly, anti-α6-immobilized proteasome (described in *Materials and Methods*) was preincubated for 10 min at 4 °C with 100 ng of Ubal or 5 μg of Ub^{VV}His or LLUb^{VV}His in a volume of 50 μL. Ub-AMC was added (to a final volume of 100 μL), and the reaction mixture was incubated for an additional 15 min at 30 °C. Reactions were terminated with SDS, and fluorescence of the released AMC was determined.

Monitoring 20S proteasome activity. Proteasomal activity was measured by monitoring the cleavage of LLVY-AMC according to the manufacturer's instructions. Briefly, 0.3 μg of purified 26S proteasome (Biomol) was preincubated at 37 °C with the indicated amounts/concentration of WT Ub, Ub^{VV}His, and MG132 for 10 min in a volume of 10 μL. LLVY-AMC was added for 10 min (to a final volume of 50 μL), the reactions were quenched by the addition of SDS, and fluorescence of the released AMC was determined.

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Fig. S1. Ub^{VV} variants have no effect on DUB activity and the 20S catalytic activity. (*A*) ^{35}S-labeled UPS substrates were subjected to ATP-dependent ubiquitination in a cell-free system. Reaction mixtures were incubated for 1 h at 37 °C (in a volume of 10 μL) in the presence of E1 and E2 (UbcH5c for Ring1B and XIAP-1, and Ubc7 for MyoD), and then for an additional hour in the presence of HeLa cell extract (50 μg in a total volume of 15 μL; as source for DUBs for all substrates and MyoD's E3). Ubal or 5 μg of purified Ub^{VV} variants was added as indicated. Proteins were resolved by SDS/PAGE (10%) and visualized by using PhosphoImager. (*B*) 26S proteasomes were isolated via immunoprecipitation from HeLa cell extract, and the DUB-associated activity was monitored by measuring the cleavage of Ub-AMC after preincubation with Ub^{VV}His, LLUb^{VV}His, or Ubal as described in *Materials and Methods*, Fig. 4*A*, and *SI Text*. (*C*) 20S proteasomal activity was monitored by using purified 26S proteasome and measuring the release of AMC from LLVY-AMC in the presence of ATP and increasing concentrations of MG132, purified Ub^{VV}His, or WT Ub as indicated. Activity was measured as described in *SI Text*.