Supplementary Methods

Synthesis of K48-Ub_2 derivatives

Synthesis of K48-Ub_2 employed mammalian E1 and E2-25K to conjugate the C-terminal glycine of UbR48 with K48 of UbD77, yielding UbR48-UbD77 [1]. Quickchange site-directed mutagenesis (Stratagene) was performed on plasmids pET-3a-Ub48R and pET-3a-UbD77 to generate Ub(R48,C8), Ub(C8,D77), Ub(A8,A44,R48), Ub(A8,A44,D77), and Ub(A8,A44,A70,D77). The mutant proteins were expressed and purified as previously described for UbR48 and UbD77, and the different K48-Ub_2 mutants were synthesized and purified accordingly [1]. Purified Ub(C8,R48)-UbD77 and UbR48-Ub(C8,D77) diubiquitins were fluorescently-labeled with Lucifer Yellow (LY) iodoacetamide [2]. Altogether, eight K48-linked diubiquitin derivatives were made in this way: Ub-Ub, $UbC8^{LY}$-Ub, Ub-$UbC8^{LY}$, Ub-Ub(A8,A44), Ub-Ub(A8,A44,A70), Ub(A8,A44)-Ub, Ub(A8,A44)-Ub(A8,A44), and Ub(A8,A44)-Ub(A8,A44,A70).

Synthesis of K11-linked and K6/K29-linked Ub_2

The enzyme E2-EPF specifically generates K11-linked polyUb chains [3]. GST-E2EPF was induced in BL21(DE3) *E. coli* cells (Novagen) for 4 h at 37 °C with 0.1 mM IPTG. Cells were pelleted, frozen, and resuspended in a buffer containing TDE (20 mM Tris, pH 7.6, 1 mM DTT, and 0.1 mM EDTA), 0.02% NP-40, and 1 mM PMSF. Cells were then lysed with 0.4 mg/ml lysozyme and DNA was digested by adding 10 mM $MgCl_2$ and 20 μg/ml DNAse I. The soluble extract was mixed for 1 h at 4 °C with glutathione-Sepharose resin equilibrated with TDE and 150 mM NaCl. The beads were put into a column and washed extensively with the same buffer. The GST-E2EPF was then eluted with the buffer containing 20 mM

reduced glutathione. The eluted protein was dialyzed in TDE and applied to a 1 ml Mono Q column equilibrated with TDE. GST-E2EPF was eluted with an 80 ml linear gradient of 0-1M NaCl in TDE. Peak fractions were pooled and exchanged into TDE by repeated concentration and dilution.

For synthesis of K11-linked Ub_2, GST-E2EPF (10 μM) was incubated with 1 mg/ml of Ub(K11R), 1 mg/ml UbD77, and 0.1 μM E1 in pH 8 conjugation buffer [1] for 30 min before the reaction was stopped with 5 mM EDTA. GST-E2EPF was removed from the mixture with glutathione-Sepharose. The unbound fraction was dialyzed against 50 mM ammonium acetate, pH 4.5, and applied to a Mono S column equilibrated in the same buffer. K11-Ub_2 was eluted with a salt gradient at ~0.33 M NaCl.

A mixture of K29-Ub_2 and K6-Ub_2 ("K6/K29-Ub_2") was prepared by conjugation of UbK0, in which all the lysines were mutated to arginines, to Ub(48R)D77 using the catalytic domain of the KIAA10 E3 ligase and the UbcH5a E2 [4].

Syntheses of ^{125}I-labeled E2-25K-(K48-linked)Ub_4 and ^{125}I-labeled Ubc13-(K63-linked)Ub_4

K48 and K63-linked polyUb tetramers, prepared as described previously [5; 6], were radioiodinated and conjugated to E2-25K or Ubc13, respectively, by autoubiquitination. First, 5 μM ^{125}I-labeled K48-Ub_4 was incubated with 15 μM E2-25K and 0.1 μM E1 for 90 min at 37°C in pH 8 conjugation buffer [1]. The reaction was stopped by adding N-ethyl-maleimide (NEM) to 4 mM and incubation for 5 min. The NEM was then consumed by adding DTT to 5 mM. The proteins were exchanged into HDE buffer (20 mM Hepes, pH 7.3, 1 mM DTT and 0.1 mM EDTA), supplemented with 0.5 mg/ml BSA, and bound to Q-Sepharose equilibrated with HDE buffer. E2-25K-(K48-linked)Ub_4 conjugates bind to the resin under

these conditions, but free, unconjugated K48-Ub_4 chains do not. The Q-Sepharose resin was then washed with HDE buffer containing 20 mM NaCl, and the E2-25K-(K48-linked)Ub_4 conjugates were eluted with HDE buffer containing 300 mM NaCl.

To generate ^{125}I-K63Ub_4-Ubc13, 5 μM ^{125}I-labeled K63-Ub_4 was incubated with 15 μM Ubc13/Mms2-His_6 heterodimer and 0.1 μM E1 for 90 min at 37 °C in conjugation buffer. The reaction was stopped as above with 4 mM NEM followed by 5 mM DTT. After this reaction, the bulk (~95%) of the conjugated ^{125}I-K63-Ub_4 was in the form of ^{125}I-K63Ub_4-Ubc13 rather than ^{125}I-K63Ub_4-Mms2-His_6. To separate the small amount of ^{125}I-K63Ub_4-Mms2-His_6 conjugate, the mixture was diluted ten-fold into Ni^{2+}-NTA binding buffer (0.1% NP40, 10 mM imidazole, 10 mM Tris, pH 8.0, 300 mM NaCl and 0.1 mg/ml BSA) and incubated for 1 h with Ni^{2+}-NTA resin equilibrated in the same buffer. The unbound fraction, which specifically contained the ^{125}I-K63Ub_4-Ubc13 conjugates, was collected, exchanged into HDE buffer, and separated from the unconjugated ^{125}I-K63-Ub_4 using Q-Sepharose as described for ^{125}I-K48Ub_4-E2-25K above.

Steady-state deubiquitination kinetics

Steady-state enzyme kinetics were measured in reactions containing 0.03 μM hOtu1 and 2.5–800 μM K48-Ub_2 in 50 mM Tris, pH 7.5, 5 mM DTT, and 0.05% BSA; incubations were at 37 °C. Aliquots of the reaction mixtures were removed at timed intervals and the K48-Ub_2 and Ub products were measured after SDS-PAGE and staining with SYPRO Ruby (Invitrogen). The velocities were then determined for each K48-Ub_2 concentration and fit with the Michaelis-Menten equation using KaleidaGraph (Synergy Software).

Ubal binding assays

hOtu1 (4.2 μM) in 50 mM Tris-Cl, pH 7.5, and 15 mM DTT was incubated with 20 μM Ubal at 0 ºC. After 15 min, adduct formation was evaluated by native PAGE (10% polyacrylamide; gel and sample loading buffers were without SDS). Gels were stained with SYPRO Ruby. To evaluate effects of free Ub on Ubal-hOtu1 complex formation, hOtu1 was preincubated for 30 min at 0 ºC with Ub prior to the incubation with Ubal.

Mass spectrometry

hOtu1-UbVS adduct was generated in the presence of Ubal, and the remaining Cys residues were alkylated with iodoacetamide (50 mM for 30 min at room temperature). The adduct and hOtu1 were separated by SDS gel electrophoresis, excised into two gel bands, and then in-gel digestion was done followed by nanoLC-MS/MS analysis using a high-resolution LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA). During trypsin digestion, Ub-VS was cleaved to a small tag (192.059 Da) on modified Cys residues. This mass shift allowed the identification of Cys23 as the Ub-VS modification site during database search.

NMR chemical shift perturbation assays

^{15}N-labeled monoUbD77 and K48-Ub_2 (uniformly ^{15}N-labeled on the distal UbR48 (Ub_2-D) or proximal UbD77 (Ub_2-P)) were prepared, purified, and dissolved in 40 mM phosphate buffer (pH 6.8) containing 7-10% D_2O and 0.02 % NaN_3. All NMR studies were performed at 23 °C on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe. NMR experiments included 2D ^{1}H-^{15}N HSQC, ^{1}H-^{15}N SOFAST-HMQC, as well as ^{15}N T_1 measurements.

NMR titrations were performed in a series of 16-18 2D HSQC/HMQC measurements on ^{15}N-labeled monoUb or K48-Ub_2 to which increasing amounts of unlabeled hOtu1C91S were added from a concentrated stock solution (in 20 mM phosphate buffer, pH 6.8, and 5 mM DTT). The titrations continued until the hOtu1/Ub_x reached a molar ratio of 2:1 (see **Supplementary Table 1**). The observed amide resonance chemical shift perturbations were calculated as follows: $\Delta\delta = [(\Delta\delta_H)^2+(\Delta\delta_N/5)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the observed chemical shift changes for ^{1}H and ^{15}N, respectively. Signal attenuation (in %) was determined as $(1-I_t/I_o)*100$, where I_o and I_t are peak intensities in the absence of hOtu1 and at a given titration step, respectively. I_t was scaled (using residues unperturbed by the addition of hOtu1C91S) in order to account for the overall loss of intensity due to increased molecular weight and dilution, as well as differences in experimental parameter settings between different measurements.

Supplementary References

1. Raasi, S. & Pickart, C. M. (2005). Ubiquitin chain synthesis. *Methods Mol Biol* **301**, 47-55.
2. Lam, Y. A., Xu, W., DeMartino, G. N. & Cohen, R. E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature* **385**, 737-40.
3. Baboshina, O. V. & Haas, A. L. (1996). Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. *J Biol Chem* **271**, 2823-31.
4. You, J. & Pickart, C. M. (2001). A HECT domain E3 enzyme assembles novel polyubiquitin chains. *J Biol Chem* **276**, 19871-8.

5. Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K. D., Cohen, R. E. & Pickart, C. M. (1997). Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. *J Biol Chem* **272**, 23712-21.

6. Hofmann, R. M. & Pickart, C. M. (2001). In vitro assembly and recognition of Lys-63 polyubiquitin chains. *J Biol Chem* **276**, 27936-43.

Table S1. NMR titration experiments.

Observed protein	Concentration (μM)		Titrant	Concentration (μM)		[L]/[P]
	Start	End		Start	End	End
^{15}N-monoUb	380	229	hOtu1C91S	0	510	2.23
^{15}N-Ub$_2$-D	240	173	hOtu1C91S	0	356	2.05
^{15}N-Ub$_2$-P	380	238	hOtu1C91S	0	481	2.02

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: **Human otubain 1 aligned with other OTU domain deubiquitinating enzymes.** (**A**) Sequence alignment of *Homo sapiens* otubain 1 (Genbank accession number NM_017670) and otubain 2 (NM_023112), *Mus musculus* otubain 1 (NM_134150), *Caenorhabditis elegans* otubain (Q9XVR6), *Saccharomyces cerevisiae* Otu1 (NC_001138), *Drosophila melanogaster* otubain (Q9VL00), and *Arabidopsis thaliana* otubain (NM_115643). The sequences were aligned with ClustalX (EMBL-EBI) and edited with ESPript software. Conserved residues are shown in red, and arrows indicate the catalytic residues; the red-highlighted boxes indicate identity among the aligned species. (**B**) Domain organization of OTU domain deubiquitinating enzymes. Numbers indicate amino acid residues. The OTU domains are shown in yellow and Zn-finger motifs are in blue.

Figure S2: **OTU domain proteins do not cleave α-linked polyUb.** Linear, α-linked Ub_5 (0.5 μg) containing five tandem repeats of Ub was incubated in 10 μl for the indicated times with 0.5 μg of hOtu1, hCezcat, ceOtu, or hOtu1ΔN41, respectively. Reaction mixtures were analyzed by SDS-PAGE followed by silver staining. As a positive control, isopeptidase T (*isoT*; 0.68 μM) was incubated with the linear Ub_5 for 1 h.

Figure S3: **Inhibition of OTU-domain DUB activities by diubiquitins and monoUb.** Cleavage of ^{125}I-radiolabeled K48-linked Ub_2 was performed in the presence of no competitor or equal, 5-fold, or 10-fold molar excess of unlabeled monoUb or Ub_2 species

of different linkage types. See Materials and Methods for details. (**A**) Inhibition of hOtu1 activity by Ub_2 and Ub. (**B**) Inhibition of hCezcat activity by Ub_2 and Ub.

Figure S4: **Steady-state kinetics of hOtu1**. hOtu1 (0.03 μM) was incubated at 37 °C with 2.5-800 μM K48-Ub_2 and products were quantified after SDS-PAGE (see Materials and Methods for details). K_m and k_{cat} were obtained by fitting the initial rates of substrate cleavage (v_0) versus substrate concentration at time-zero, $[K48\text{-}Ub_2]_0$; fitted values and standard errors are shown in the *inset*.

Figure S5: **Free Ub has a negligible effect on Ubal binding to hOtu1.**. Ubal binding to hOtu1 was evaluated by native gel electrophoresis as described in the **Supplementary Methods**. Even when added to 2 mM, a 100-fold molar excess over Ubal, Ub had no effect on the amount of hOtu1-Ubal complex. Samples in the first three lanes contained, respectively, only Ub, Ubal (20 μM), or hOtu1 (4.2 μM)

Figure S6: **Identification of hOtu1 residue C23 as the primary site of adduct formation with UbVS.**. *Left panel:* Formation of hOtu1 adduct with HAUbVS in the presence of Ubal (lane 3). Lane 1 shows Otu1 with HAUbVS alone and lane 2, Otu1 with HAUbVS and Ub. The asterisk indicates nonspecific protein contaminants (i.e. keratins). The adduct-containing gel band was excised, digested by trypsin, and analyzed by LC-MS/MS. *Right panel:* The modified peptide was identified as QEPLGSDSEGVNC#LAYDEAIM$AQQDR, where "C#" indicates UbVS-modified cysteine-23, and "M$" indicates oxidized methionine (i.e., methionine sulfoxide). Shown

is a comparison of peptide ions detected in the adduct (*upper* spectrum) and hOtub1 (*lower* spectrum) by LC-MS/MS. Both spectra show the main ions eluted from reverse-phase chromatography during 1 min (77.8-78.8 min) of the LC gradient. As expected, the majority of peptide ions were shared in the two samples (*e.g.*, 449.58 *m/z*, 673.87 *m/z*, 862.49 *m/z*, 1149.99 *m/z*). The modified peptide was detected as a triply-charged ion (1016.80 *m/z*) as indicated. In parallel, the hOtu1 was also analyzed and the same peptide without UbVS modification was found as a triply-charged ion (971.77 *m/z*) and as a doubly-charged ion (1457.15 *m/z*).

Figure S1:

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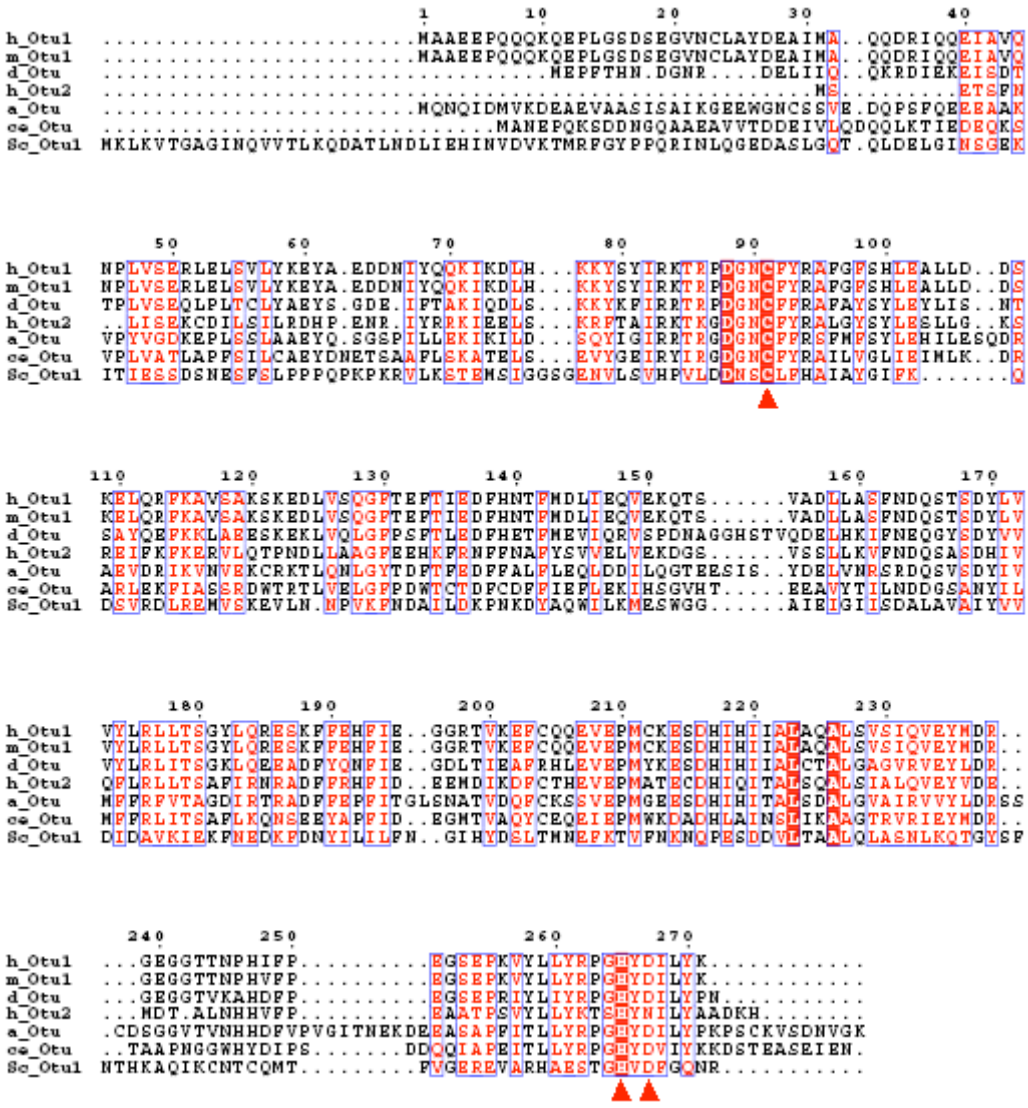

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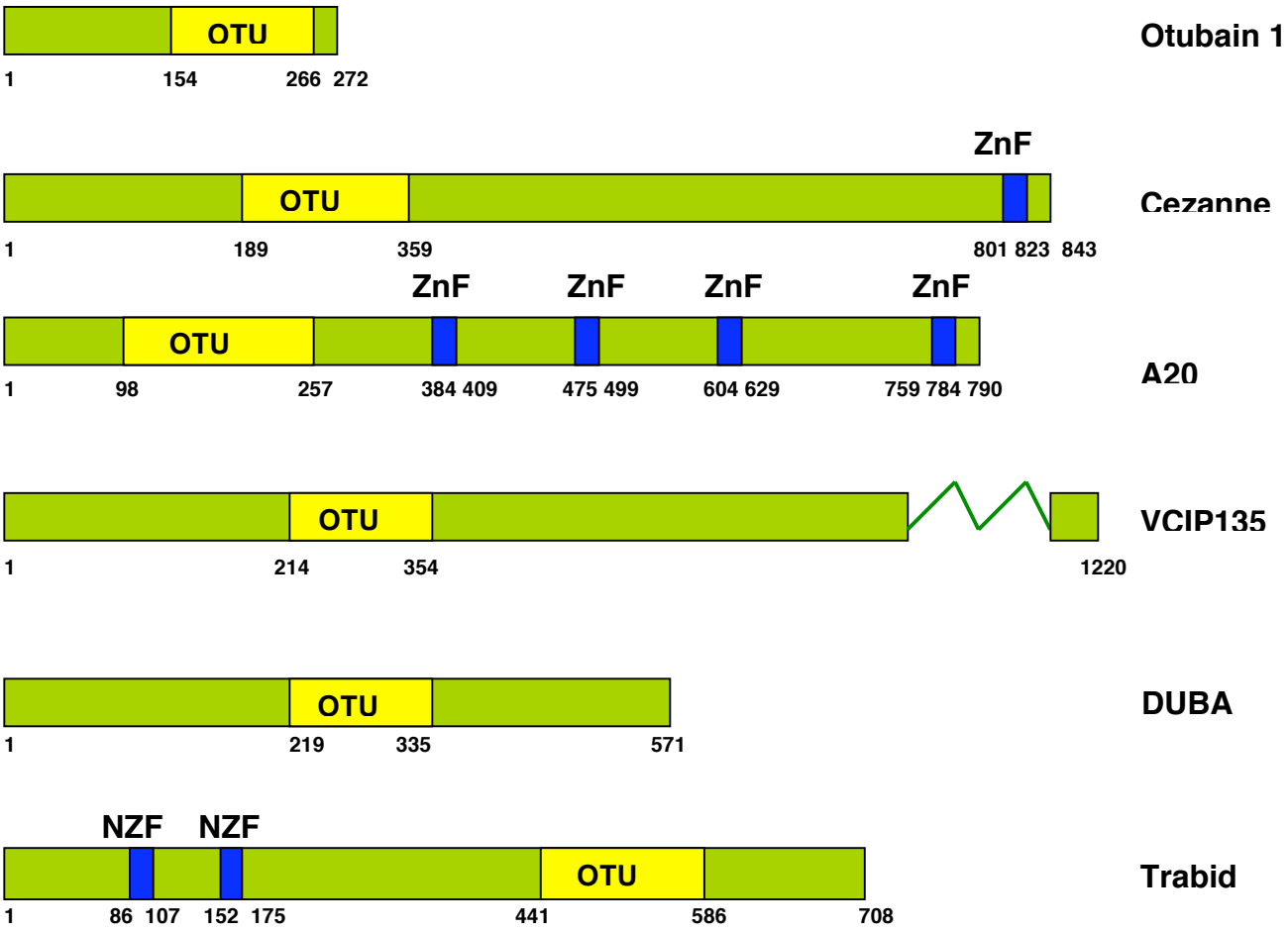
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154
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272
Otubain 1
ZnF
OTU
1
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843
Cezanne
ZnF
ZnF
ZnF
ZnF
OTU
1
98
257
384
409
475
499
604
629
759
784
790
A20
OTU
1
214
354
1220
VCIP135
OTU
1
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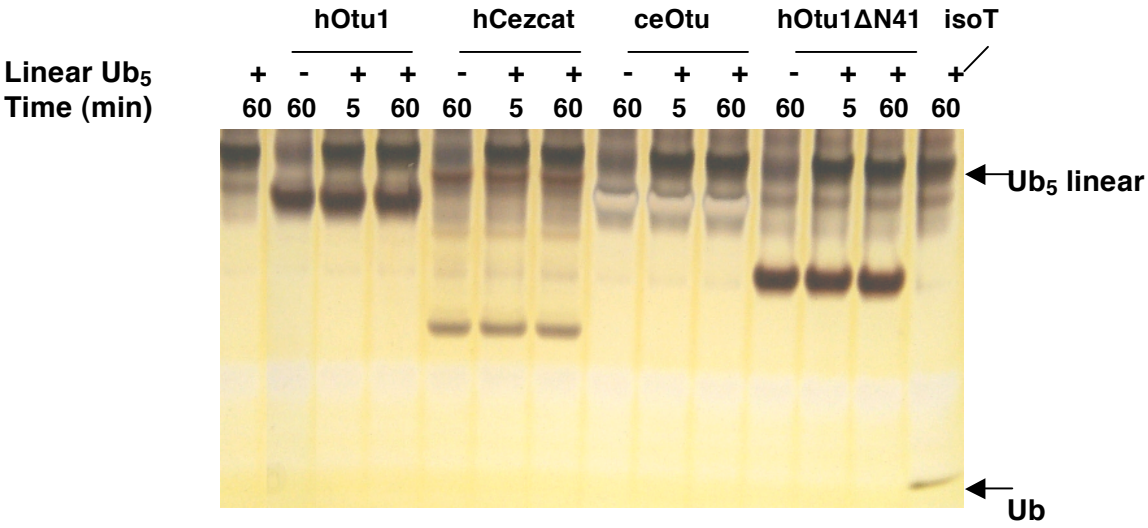
708
Trabid

Figure S2


hOtu1
hCezcat
ceOtu
hOtu1ΔN41
isoT
Linear Ub5
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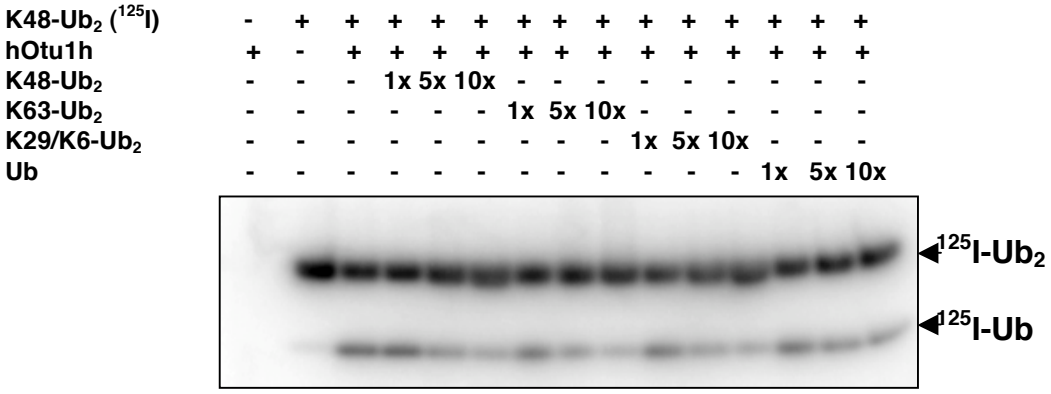
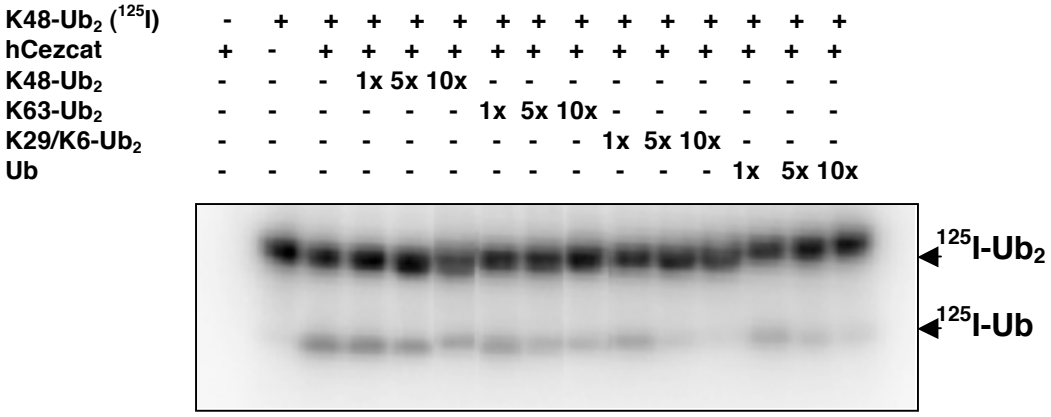
Ub5 linear
Ub

Figure S3

(A)


K48-Ub2 (125I) - + + + + + + + + + + + + + +
hOtu1h + - + + + + + + + + + + + + +
K48-Ub2 - - - 1x 5x 10x - - - - - - - - -
K63-Ub2 - - - - - - 1x 5x 10x - - - - - -
K29/K6-Ub2 - - - - - - - - - 1x 5x 10x - - -
Ub - - - - - - - - - - - - 1x 5x 10x
125I-Ub2
125I-Ub

(B)


K48-Ub2 (125I) - + + + + + + + + + + + + + +
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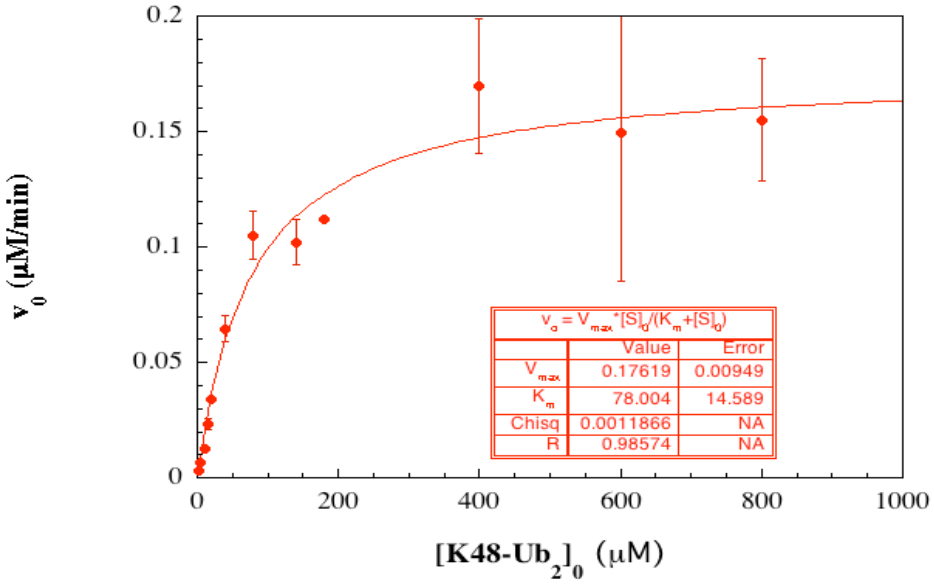
K48-Ub2 - - - 1x 5x 10x - - - - - - - - -
K63-Ub2 - - - - - - 1x 5x 10x - - - - - -
K29/K6-Ub2 - - - - - - - - - 1x 5x 10x - - -
Ub - - - - - - - - - - - - 1x 5x 10x
125I-Ub2
125I-Ub

Figure S4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400
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800
1000
v0 (μM/min)
[K48-Ub2]0 (μM)
v0 = Vmax*[S]0/(Km+[S]0)
Value
Error
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Km
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Chisq
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R
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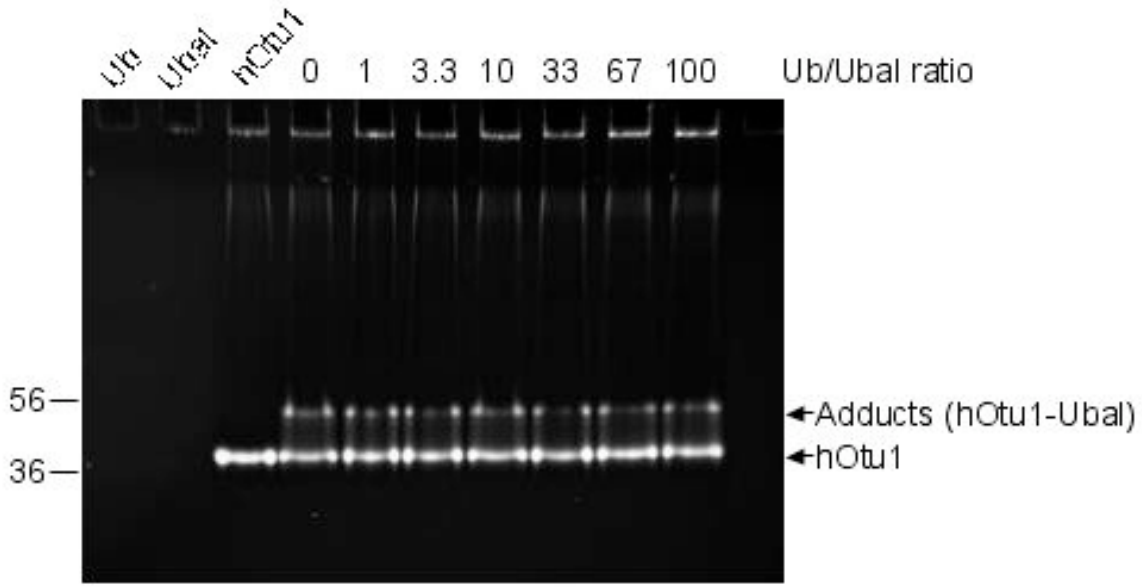
NA

Figure S5


Ub
Ubal
hOtu1
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100
Ub/Ubal ratio
56
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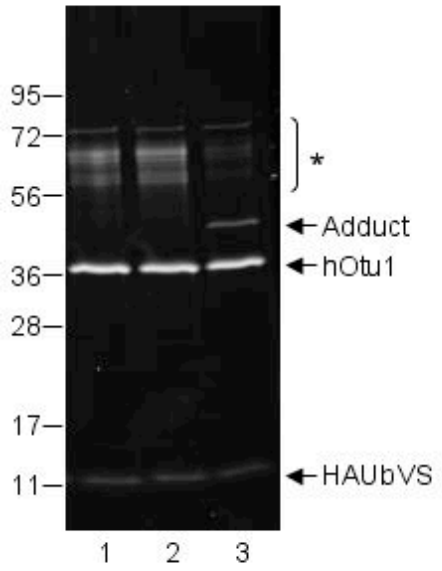
Adducts (hOtu1-Ubal)
hOtu1

Figure S6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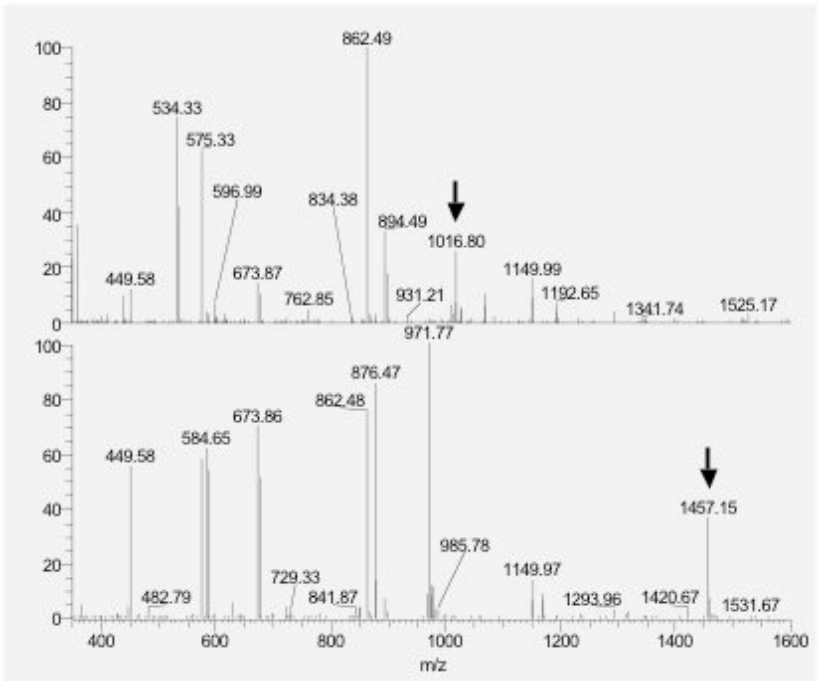
2
3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m/z