Molecular Cell

Supplemental Information

Proteasome-Bound UCH37 Debranched Ubiquitin Chains to Promote Degradation

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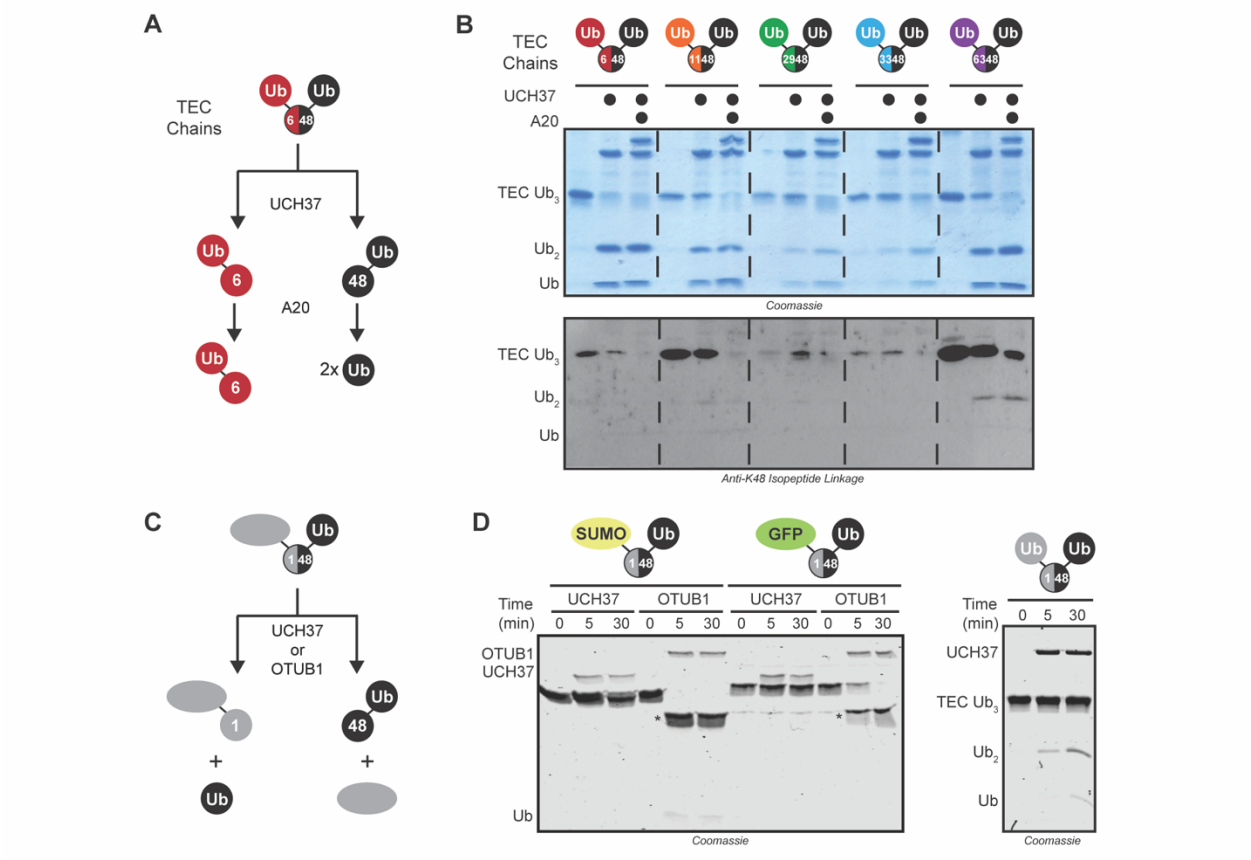


FIGURE S1. Linkage Selectivity of UCH37 with TEC Trimers, Related to Figure 1
(A) Schematic showing the sequential DUB assay used to assess the linkage selectivity of UCH37 with thiol-ene coupling (TEC)-derived Ub trimers.
(B) SDS-PAGE analysis of the sequential digests of K48 containing branched tri-Ub with UCH37 (5 μM) followed by A20 (5 μM) for 1 h each. The linkage of the di-Ub species was visualized using the anti-Ub K48-selective antibody.
(C) Schematic for assessing the hydrolytic activity of UCH37 or OTUB1 with native SUMO or GFP di-Ub fusion and TEC tri-Ub.
(D) SDS-PAGE analysis of native M1/K48 SUMO di-Ub and M1/K48 GFP di-Ub (10 μM, left) and TEC M1/K48 tri-Ub (10 μM, right) by UCH37 or OTUB1 (1 μM).
* indicates the cleavage products of either SUMO-Ub or GFP-Ub

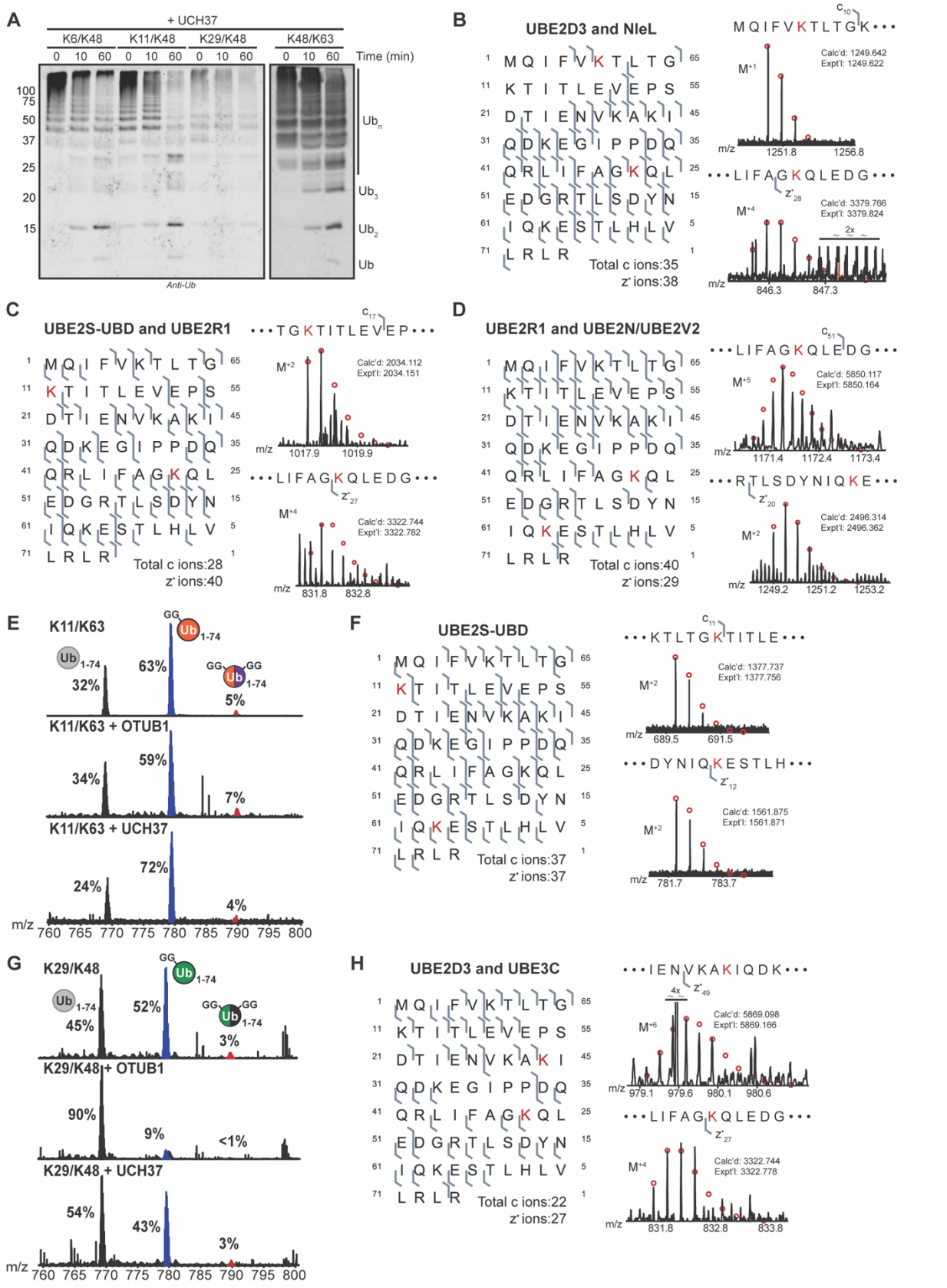

A
+ UCH37
K6/K48
K11/K48
K29/K48
K48/K63
0 10 60 0 10 60 0 10 60 0 10 60
Time (min)
100
75
50
37
25
20
15
Ub$_n$
Ub$_3$
Ub$_2$
Ub
Anti-Ub
B
UBE2D3 and NleL
M Q I F V K T L T G
K T I T L E V E P S
D T I E N V K A K I
Q D K E G I P P D Q
Q R L I F A G K Q L
E D G R T L S D Y N
I Q K E S T L H L V
L R L R
Total c ions:35
z˙ ions:38
c10
M Q I F V K T L T G K • • •
Calc'd: 1249.642
Expt'l: 1249.622
M+1
m/z
1251.8
1256.8
• • • L I F A G K Q L E D G • • •
z˙28
Calc'd: 3379.766
Expt'l: 3379.824
M+4
2x
m/z
846.3
847.3
C
UBE2S-UBD and UBE2R1
Total c ions:28
z˙ ions:40
c17
• • • T G K T I T L E V E P • • •
Calc'd: 2034.112
Expt'l: 2034.151
M+2
m/z
1017.9
1019.9
• • • L I F A G K Q L E D G • • •
z˙27
Calc'd: 3322.744
Expt'l: 3322.782
M+4
m/z
831.8
832.8
D
UBE2R1 and UBE2N/UBE2V2
Total c ions:40
z˙ ions:29
c51
• • • L I F A G K Q L E D G • • •
Calc'd: 5850.117
Expt'l: 5850.164
M+5
m/z
1171.4
1172.4
1173.4
• • • R T L S D Y N I Q K E • • •
z˙20
Calc'd: 2496.314
Expt'l: 2496.362
M+2
m/z
1249.2
1251.2
1253.2
E
K11/K63
Ub1-74
GG
63%
32%
5%
K11/K63 + OTUB1
59%
34%
7%
K11/K63 + UCH37
72%
24%
4%
m/z
760 765 770 775 780 785 790 795 800
F
UBE2S-UBD
Total c ions:37
z˙ ions:37
c11
• • • K T L T G K T I T L E • • •
Calc'd: 1377.737
Expt'l: 1377.756
M+2
m/z
689.5
691.5
• • • D Y N I Q K E S T L H • • •
z˙12
Calc'd: 1561.875
Expt'l: 1561.871
M+2
m/z
781.7
783.7
G
K29/K48
52%
45%
3%
K29/K48 + OTUB1
90%
9%
<1%
K29/K48 + UCH37
54%
43%
3%
m/z
760 765 770 775 780 785 790 795 800
H
UBE2D3 and UBE3C
Total c ions:22
z˙ ions:27
• • • I E N V K A K I Q D K • • •
4x
z˙49
Calc'd: 5869.098
Expt'l: 5869.166
M+6
m/z
979.1
979.6
980.1
980.6
• • • L I F A G K Q L E D G • • •
z˙27
Calc'd: 3322.744
Expt'l: 3322.778
M+4
m/z
831.8
832.8
833.8

FIGURE S2. Analysis HMW Chain Cleavage with UCH37, Related Figure 2.
(A) Western blot analysis of UCH37-catalyzed cleavage with HMW K6/K48, K11/K48, K29/K48, and K48/K63 Ub chains. Hydrolysis of ubiquitin chains is tracked by SDS-PAGE and visualized using the α-Ub P4D1 antibody.
(B-D) Observed ETD fragments (c and z· ions) mapped onto the Ub sequence containing a di-Gly modification at the following positions: K6 and K48 (B), K11 and K48 (C), and K48 and K63 (D).
(E) Ub MiD MS analysis of HMW K11/K63 chains (top) treated with either OTUB1 (1 μM, middle) or UCH37 (1 μM, bottom) for 1 h. Percentages correspond to the relative quantification values of the 11+ charge state for each Ub species: Ub_{1-74}, 1xdiGly-Ub_{1-74}, and 2xdiGly-Ub_{1-74}.
(F) Observed ETD fragments (c and z· ions) mapped onto the Ub sequence containing a di-Gly modification at K11 and K63.
(G) Ub MiD MS analysis of HMW K29/K48 chains (top) treated with either OTUB1 (1 μM, middle) or UCH37 (1 μM, bottom) for 1 h. Percentages correspond to the relative quantification values of the 11+ charge state for each Ub species: Ub_{1-74}, 1xdiGly-Ub_{1-74}, and 2xdiGly-Ub_{1-74}.
(H) Observed ETD fragments (c and z· ions) mapped onto the sequence of Ub containing a di-Gly modification at K29 and K48.
ETD fragments show the presence of a di-Gly modification (B-D, F, and H) at each respective lysine position labeled in red. Red circles represent theoretical isotopic abundance distributions of isotopomer peaks. Calc'd: calculated monoisotopic weight; expt'l: experimental monoisotopic weight.

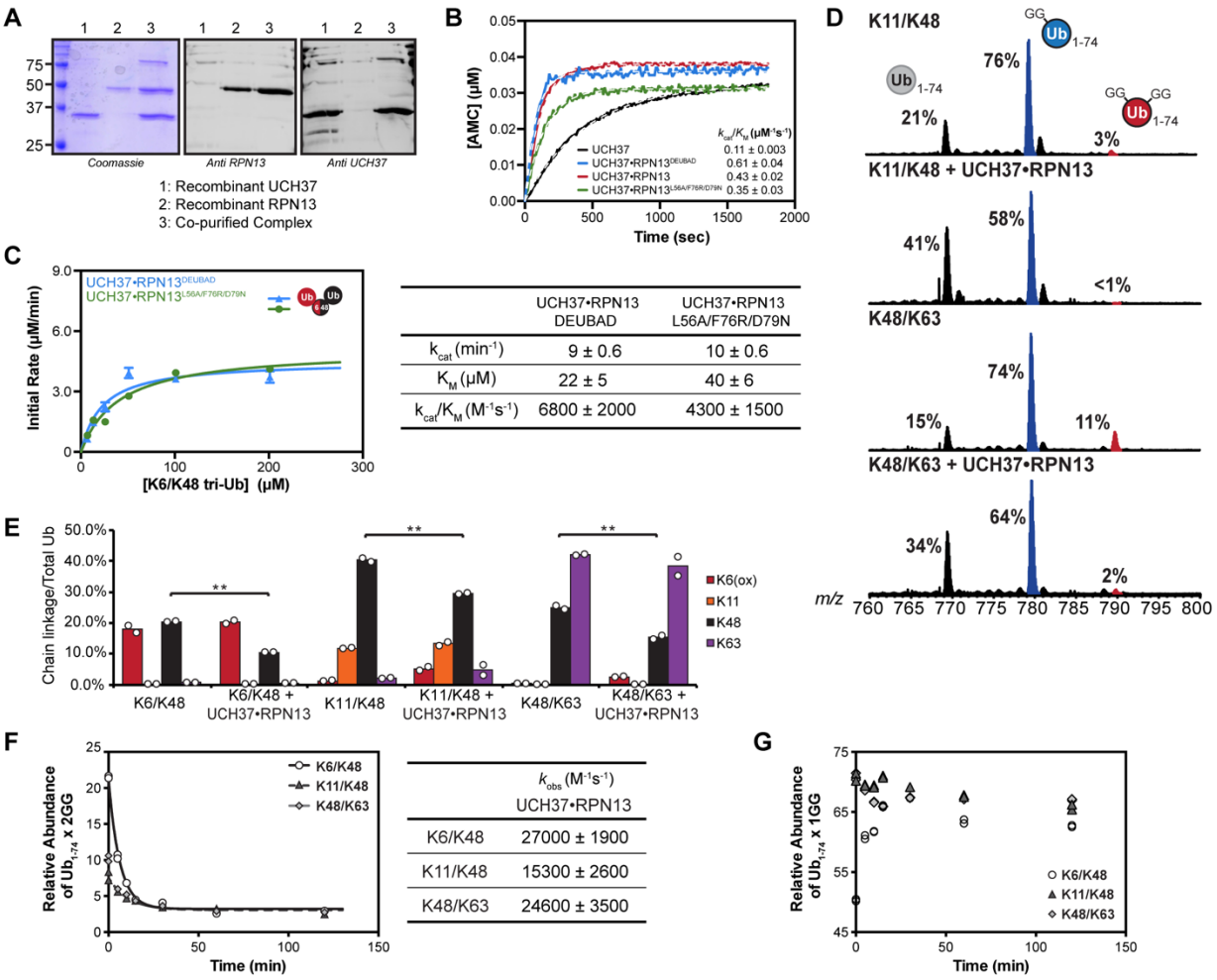


FIGURE S3. Effects of RPN13 on Debranching Activity, Related to Figure 3

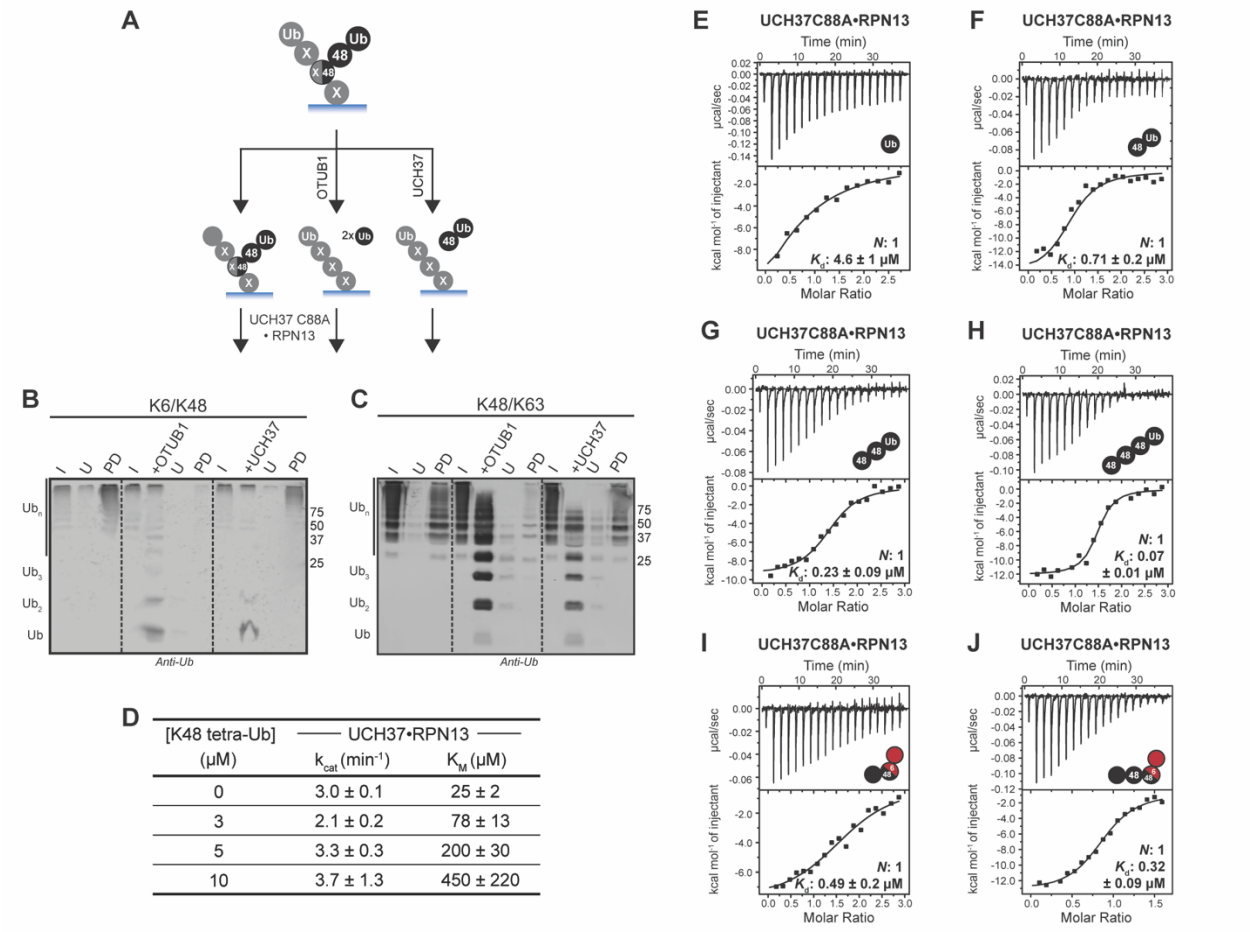
(A) Characterization of the UCH37•RPN13 co-purified complex: Coomassie gel (left), α-RPN13 immunoblot (middle), and α-UCH37 immunoblot (right). 1 = recombinant UCH37, 2 = recombinant RPN13, 3 = co-purified complex.

(B) Ubiquitin-AMC hydrolysis of UCH37, UCH37•RPN13^{DEUBAD}, UCH37•RPN13, and UCH37•RPN13$^{L56A/F76R/D79N}$ (20 nM). All curves are representative traces and fits are derived from averaging two independent experiments to pseudo first-order kinetics: $Y = Y_{max}(1\text{-}e^{(kcat/Km)\cdot E_o\cdot t})$.

(C) Michaelis-Menten plot for the hydrolysis of native K6/K48 branched tri-Ub by either UCH37•RPN13^{DEUBAD} or UCH37•RPN13$^{L56A/F76R/D79N}$ (0.5 μM, left). Table of kinetic parameters were measured for all experiments following the initial rates of di-Ub formation (right). All kinetic curves are representative traces and constants are derived from averaging fits of independent experiments with SD (n = 3).

(D) Ub MiD MS analysis of HMW K11/K48 chains (top) and HMW K48/K63 chains (bottom) treated with UCH37•RPN13 (1 μM) for 1 h. Percentages correspond to the relative quantification values of the 11+ charge state for each Ub species: $Ub_{1\text{-}74}$, 1xdiGly-$Ub_{1\text{-}74}$, and 2xdiGly-$Ub_{1\text{-}74}$.

(E) Ub-AQUA analysis of HMW K6/K48, K11/K48, and K48/K63 chains before and after UCH37•RPN13 (1 μM) treatment for 1 h. For all points, *P<0.025, **P<0.01 (Student's T-test). Quantification values are derived from averaging fits of 2 independent experiments shown with SEM.
(F-G) Steady-state parameters for the hydrolysis of HMW K6/K48, K11/K48, and K48/K63 chains by UCH37•RPN13 (0.5 μM, left). Catalytic efficiencies (k_{cat}/K_m) are calculated from the 11+ charge state of the 2xdiGly-Ub_{1-74} species. Table of kinetic parameters (right) were measured for all experiments following the first-order decay rates of the 2xdiGly-Ub_{1-74} species (F) or tracking the changes of the 1xdiGly-Ub_{1-74} species (G) over time. All kinetic curves are representative traces and constants are derived from averaging fits of independent experiments with SD (n = 2).



D

[K48 tetra-Ub] (µM)	UCH37•RPN13 k_{cat} (min^{-1})	UCH37•RPN13 K_M (µM)
0	3.0 ± 0.1	25 ± 2
3	2.1 ± 0.2	78 ± 13
5	3.3 ± 0.3	200 ± 30
10	3.7 ± 1.3	450 ± 220

FIGURE S4. UCH37•RPN13 Binding Data, Related to Figure 4

(A) Schematic for pull-downs with mixed K48-linked HMW chains.

(B-C) Western blot analysis of pull-downs with HMW K6/K48 (B) and K48/K63 (C) chains. HMW chains (12 µg) are either untreated or pre-treated with OTUB1 (1 µM) or UCH37 (2 µM) for 1 h and then captured using immobilized His-tagged RPN13•UCH37 C88A (5 nmol). Gels are labeled as I = HMW chain input, U = unbound Ub chains, and PD = pull-down enrichment of Ub chains.

(D) Kinetic constants derived from Michaelis-Menten analysis for the hydrolysis of native K6/K48 branched tri-Ub by UCH37•RPN13 (0.5 µM) in the presence of K48 tetra-Ub.

(E-J) ITC analysis of UCH37C88A•RPN13 binding to mono-Ub (E), K48-linked di-Ub (F), tri-Ub (G), tetra-Ub (H), K6/K48 branched tri-Ub (I) and K6/K48 branched tetra-Ub (J). The reported K_ds are derived from averaging fits of two independent experiments.

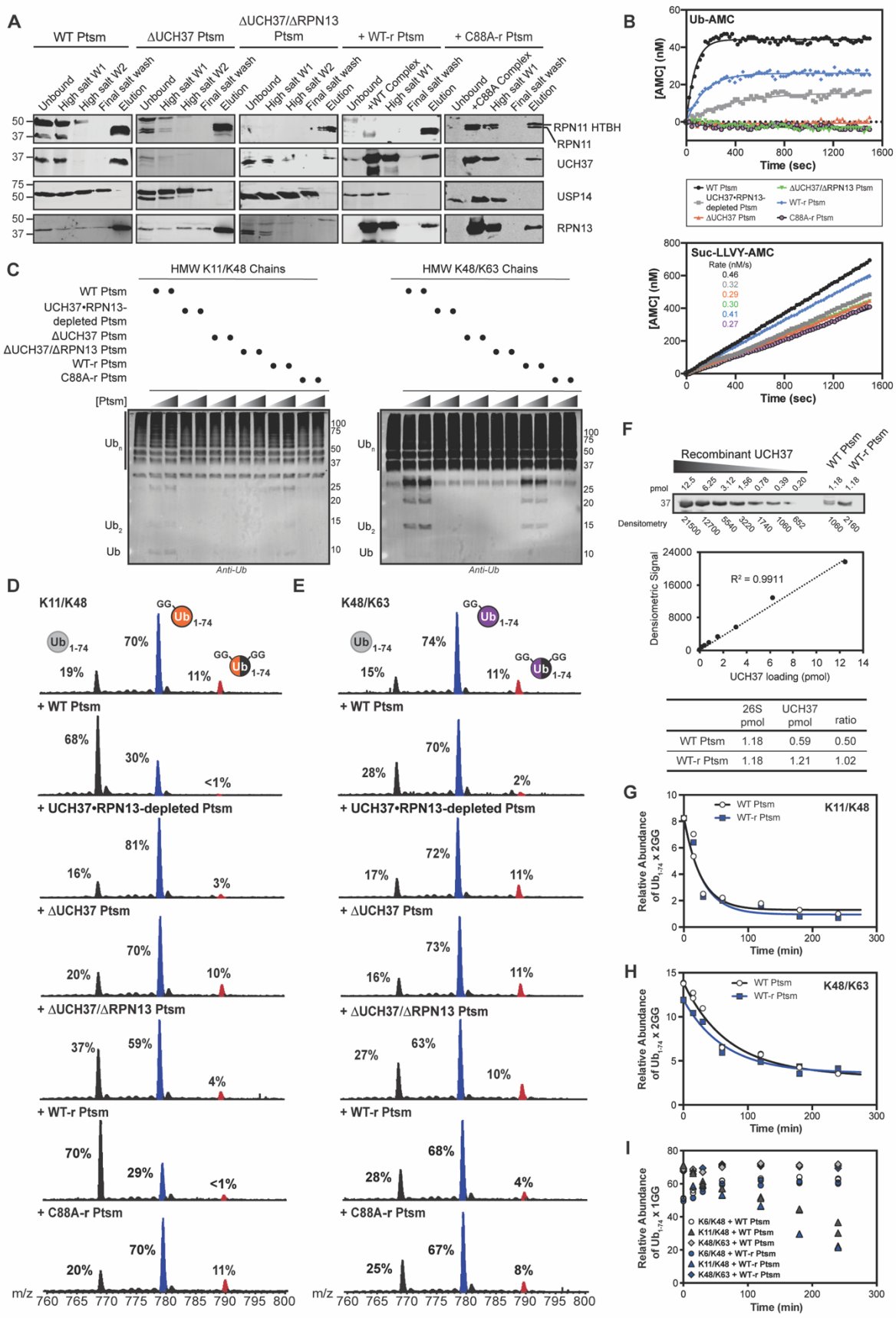

A
WT Ptsm
ΔUCH37 Ptsm
ΔUCH37/ΔRPN13 Ptsm
+ WT-r Ptsm
+ C88A-r Ptsm
Unbound
High salt W1
High salt W2
Final salt wash
Elution
+WT Complex
+C88A Complex
RPN11 HTBH
RPN11
UCH37
USP14
RPN13
B
Ub-AMC
[AMC] (nM)
Time (sec)
WT Ptsm
UCH37•RPN13-depleted Ptsm
ΔUCH37 Ptsm
ΔUCH37/ΔRPN13 Ptsm
WT-r Ptsm
C88A-r Ptsm
Suc-LLVY-AMC
Rate (nM/s)
0.46
0.32
0.29
0.30
0.41
0.27
C
HMW K11/K48 Chains
HMW K48/K63 Chains
[Ptsm]
Ub
Ub2
Ubn
Anti-Ub
D
K11/K48
E
K48/63
+ WT Ptsm
+ UCH37•RPN13-depleted Ptsm
+ ΔUCH37 Ptsm
+ ΔUCH37/ΔRPN13 Ptsm
+ WT-r Ptsm
+ C88A-r Ptsm
m/z
F
Recombinant UCH37
pmol
Densitometry
Densiometric Signal
R² = 0.9911
UCH37 loading (pmol)
26S pmol
UCH37 pmol
ratio
WT Ptsm 1.18 0.59 0.50
WT-r Ptsm 1.18 1.21 1.02
G
K11/K48
Relative Abundance of Ub1-74 x 2GG
Time (min)
H
K48/K63
I
Relative Abundance of Ub1-74 x 1GG
K6/K48 + WT Ptsm
K11/K48 + WT Ptsm
K48/K63 + WT Ptsm
K6/K48 + WT-r Ptsm
K11/K48 + WT-r Ptsm
K48/K63 + WT-r Ptsm

FIGURE S5. UCH37 is Required for Debranching by the Proteasome, Related to Figure 5.

(A) Western blot analysis showing the loss of USP14 during purification. For KO cell lines the loss of UCH37 or RPN13 are also observed. For replenish experiments, addition of recombinant UCH37 and RPN13 are found in the final elution.

(B) Ubiquitin-AMC (top) and Suc-LLVY-AMC (bottom) hydrolysis of each indicated proteasome (1 μg). All curves are representative traces and fits are derived from averaging two independent experiments.

(C) Western blot analysis of HMW K11/K48 (left) and K48/K63 (right) chain debranching for 2 h with increasing concentration of Ptsms (2 and 5 μg) using the α-Ub P4D1 antibody.

(D-E) Ub MiD MS analysis of HMW K11/K48 (D) and K48/K63 (E) chains subjected to each indicated Ptsm complex (10 μg) for 4 h. Percentages correspond to the relative quantification values of the 11+ charge state for each Ub species: Ub_{1-74}, 1xdiGly-Ub_{1-74}, and 2xdiGly-Ub_{1-74}.

(F) Quantitative western blot analysis to determine concentration of UCH37 in WT and UCH37•RPN13-replenished proteasomes for kinetic analysis of cleavage reactions.

(G-H) Steady-state parameters for the hydrolysis of HMW K11/K48 (G) and K48/K63 (H) chains by WT proteasome (10 μg, black) and UCH37•RPN13-replenished proteasome (10 μg, blue). Catalytic efficiencies (k_{cat}/K_m) are calculated from the 11+ charge state of the 2xdiGly-Ub_{1-74} species. All curves are averaged representative traces from averaging fits of independent experiments with SD (n = 2).

(I) Steady-state parameters for the changes in the 1xdiGly-Ub_{1-74} species from HMW chains by WT proteasome (10 μg, black) and UCH37•RPN13-replenished proteasome (10 μg, blue). All curves are averaged representative traces from averaging fits of independent experiments with SD (n = 2).

*Ptsms are labeled as wild-type = WT, displaced by RPN2 peptide = UCH37•RPN13-depleted, UCH37 knock-out = ΔUCH37, RPN13 knock-out = ΔUCH37/ΔRPN13, UCH37•RPN13-replenished = WT-r (UCH37•RPN13) and C88A-r (UCH37 C88A•RPN13)

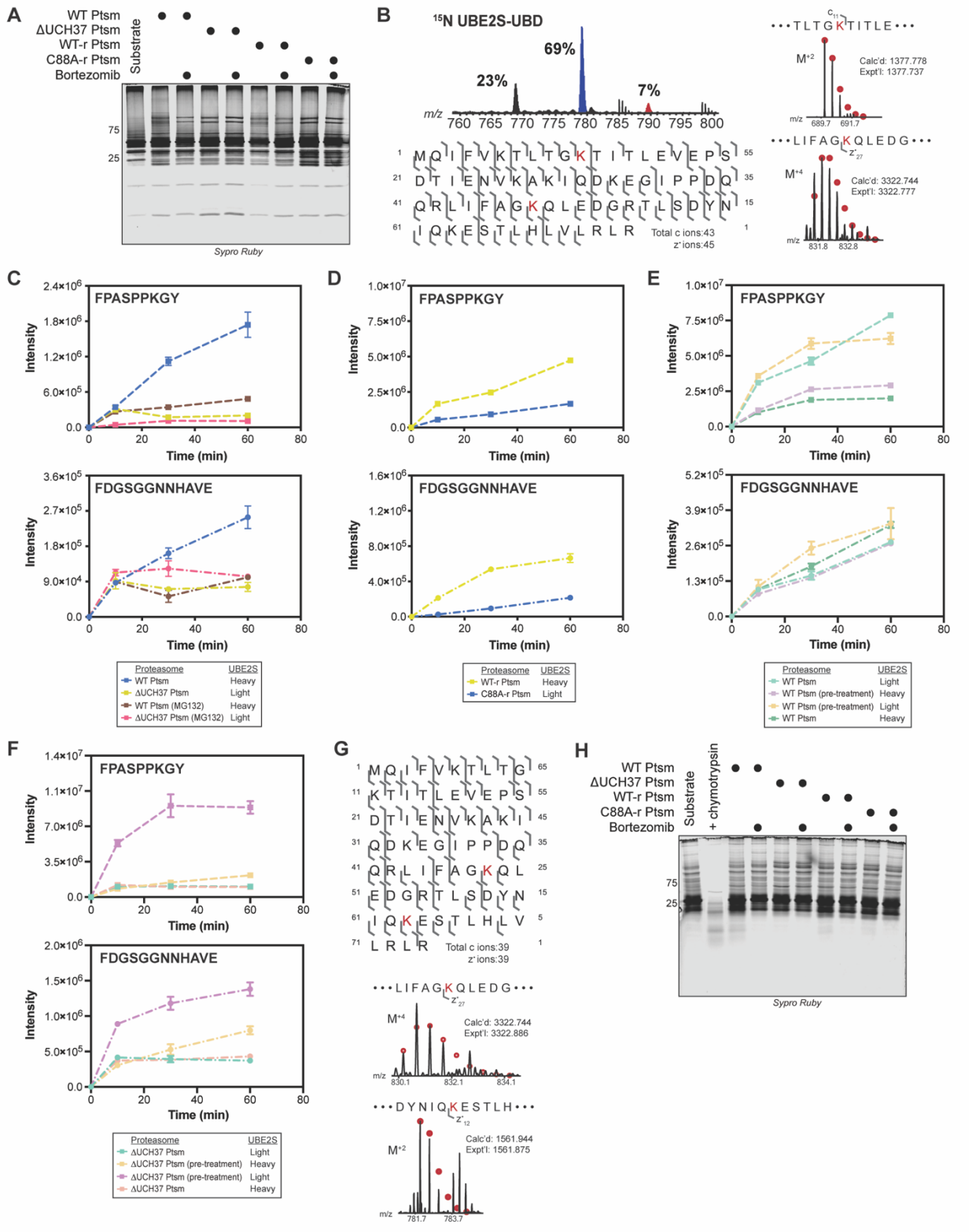

A
WT Ptsm
ΔUCH37 Ptsm
WT-r Ptsm
C88A-r Ptsm
Bortezomib
Substrate
75
25
Sypro Ruby
B
15N UBE2S-UBD
69%
23%
7%
m/z
760 765 770 775 780 785 790 795 800
Total c ions:43
z˙ ions:45
M+2
Calc'd: 1377.778
Expt'l: 1377.737
M+4
Calc'd: 3322.744
Expt'l: 3322.777
C
D
E
FPASPPKGY
FDGSGGNNHAVE
Intensity
Time (min)
Proteasome
UBE2S
WT Ptsm
Heavy
ΔUCH37 Ptsm
Light
WT Ptsm (MG132)
Heavy
ΔUCH37 Ptsm (MG132)
Light
WT-r Ptsm
Heavy
C88A-r Ptsm
Light
WT Ptsm
Light
WT Ptsm (pre-treatment)
Heavy
WT Ptsm (pre-treatment)
Light
WT Ptsm
Heavy
F
ΔUCH37 Ptsm
Light
ΔUCH37 Ptsm (pre-treatment)
Heavy
ΔUCH37 Ptsm (pre-treatment)
Light
ΔUCH37 Ptsm
Heavy
G
Total c ions:39
z˙ ions:39
M+4
Calc'd: 3322.744
Expt'l: 3322.886
M+2
Calc'd: 1561.944
Expt'l: 1561.875
H
WT Ptsm
ΔUCH37 Ptsm
WT-r Ptsm
C88A-r Ptsm
Bortezomib
Substrate
+ chymotrypsin
75
25
Sypro Ruby

FIGURE S6. Debranching Regulates Proteasomal Degradation, Related to Figure 6.

(A) Total protein stain (SYPRO® Ruby) for the degradation of K11/K48-Ub_n-UBE2S-UBD after 1 h using the indicated Ptsm complexes (5 μg) with and without the presence of bortezomib (1 μM).

(B) Ub MiD MS analysis of K11/K48-Ub_n-^{15}N UBE2S-UBD (top). Percentages correspond to the relative quantification values of the 11+ charge state for each Ub species: Ub_{1-74}, 1xdiGly-Ub_{1-74}, and 2xdiGly-Ub_{1-74}. Observed ETD fragments (c and z· ions, bottom) mapped onto the Ub sequence containing a di-Gly modification at K11 and K48.

(C-F) PRM MS analysis of UBE2S-UBD peptides formed by Ptsms. The UBE2S peptide FPASPPKGY is shown on the top and the UBD peptide FDGSGGNNHAVE is on the bottom. (C) Heavy and light UBE2S-UBD were mixed with either WT Ptsm (5 μg) or ΔUCH37 Ptsm (5 μg), respectively, in the presence and absence of MG132 (10 μM). (D) Light and heavy UBE2S-UBD were mixed with either WT-r Ptsm (5 μg) or C88A-r Ptsm (5 μg). (E) Light and heavy UBE2S-UBD are either untreated or pre-treated for 1 h with UCH37•RPN13 (1 μM) and then mixed with WT Ptsm (5 μg). (F) Light and heavy UBE2S-UBD are either untreated or pre-treated with UCH37•RPN13 (1 μM) for 1 h and then mixed with ΔUCH37 Ptsm (5 μg).

(G) Observed ETD fragments (c and z· ions, bottom) for the ubiquitinated titin-$I27^{V15P}$-23-K-35 substrate mapped onto the Ub sequence containing a di-Gly modification at K48 and K63.

(H) Total protein stain (SYPRO® Ruby) for the degradation of K48/K63-Ub_n-titin-$I27^{V15P}$-23-K-35 after 1 h using the indicated Ptsm complexes (5 μg) with and without the presence of bortezomib (1 μM).

ETD fragments show the presence of a di-Gly modification (B and F) at each respective lysine position labeled in red. Red circles represent theoretical isotopic abundance distributions of isotopomer peaks. Calc'd: calculated monoisotopic weight; expt'l: experimental monoisotopic weight. All MS curves are representative depictions from the sum of the transition ions of each monitored peptide (C-F) with a dashed line connecting the averaging of independent experiments with SD (n = 4). All fluorescence histograms are representative traces from averaging fits of three independent experiments.

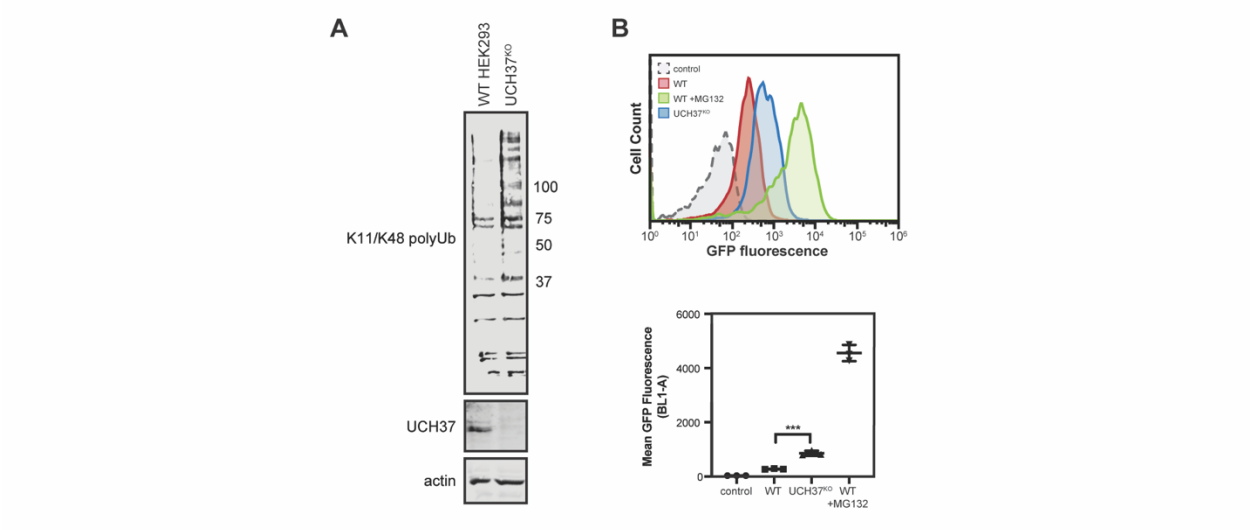


FIGURE S7. UCH37 Enhances Proteasomal Degradation, Related to Figure 7.
(A) Steady-state levels of Ub conjugates were assessed in WT and UCH37^{KO} cells using the indicated antibodies. This is a representative blot of a biological replicate.
(B) Fluorescence histograms of control HEK293, WT HEK293 GFP^{u} (treated with and without MG132), and UCH37^{KO} GFP^{u} cells. Quantitation of mean GFP fluorescence is from the averaging of three independent experiments. ***P<0.0005 (Student's t-test).