Supporting information

Ubiquitin Chain Enrichment Middle-Down Mass Spectrometry (UbiChEM-MS) Enables Characterization of Branched Ubiquitin Chains *in cellulo*

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I. Supplemental Figures and Tables

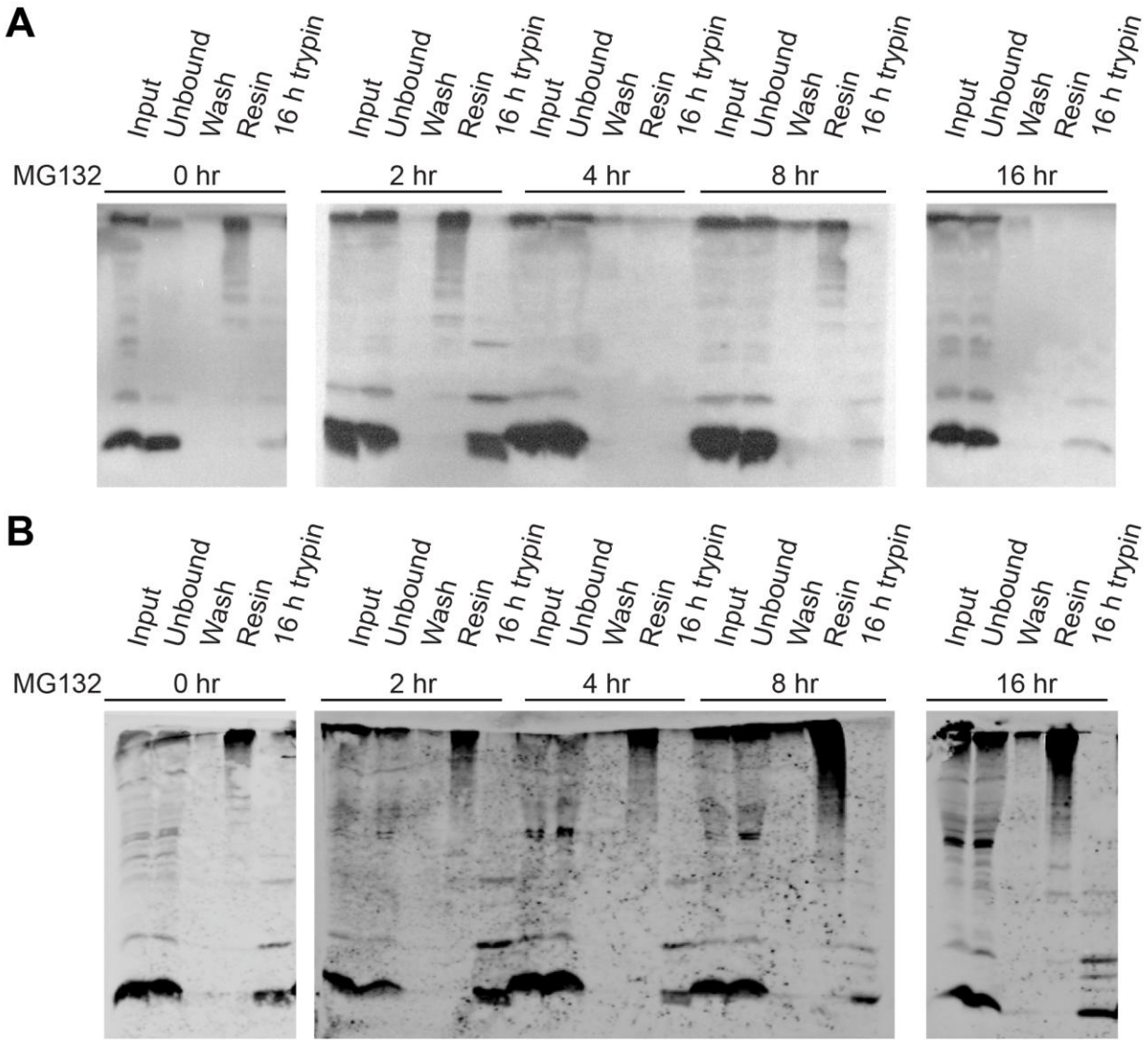


Figure S1. Ub chains isolated from cells treated with MG132 at different time points. HEK cells were treated with the proteasome inhibitor MG132 (10 μM) for indicated times prior to harvest and lysis. Lysate was then incubated with **A.** agarose TUBEs and **B.** Halo-NZF1 resin overnight (16hours) at 4^{o}C and subjected to minimal trypsinolysis described in Table S1. Samples were separated on a 15 % SDS-PAGE gel and then analyzed by western blot with anti-Ub antibody (P4D1).

Table S1. Empirically determined optimal ratios of cell lysate to trypsin used throughout this study.

Treatment	Agarose TUBE2 (µL)	Halo-NZF1 resin (µL)	Input lysate (mg)	Trypsin (µg)	Ratio lysate to trypsin (mg:µg)
None	100	-	50	2.5	20:1
None	-	200	45	7.5	18:1
MG132	100	-	50	7.5	6.7:1
MG132	-	200	45	7.5	6:1

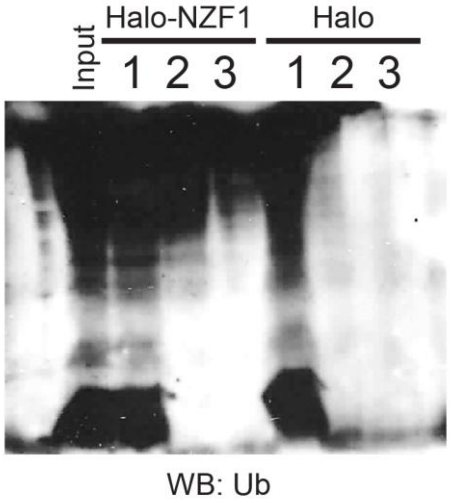


Figure S2. Halo-NZF1 resin can pull down ubiquitinated proteins from cell lysate while Halo resin alone does not. Lysate from HEK cells treated with MG132 was incubated with HaloLink (Promega) resin modified with either the Halo-NZF1 fusion protein or the Halo protein alone. 1. Flow through 2. Aliquot of the resin after wash with lysis buffer (see experimental section) 3. Aliquot of supernatant after elution with 0.2M Glycine pH2.5. Samples were analyzed by western blot using anti-Ub antibody (P4D1).

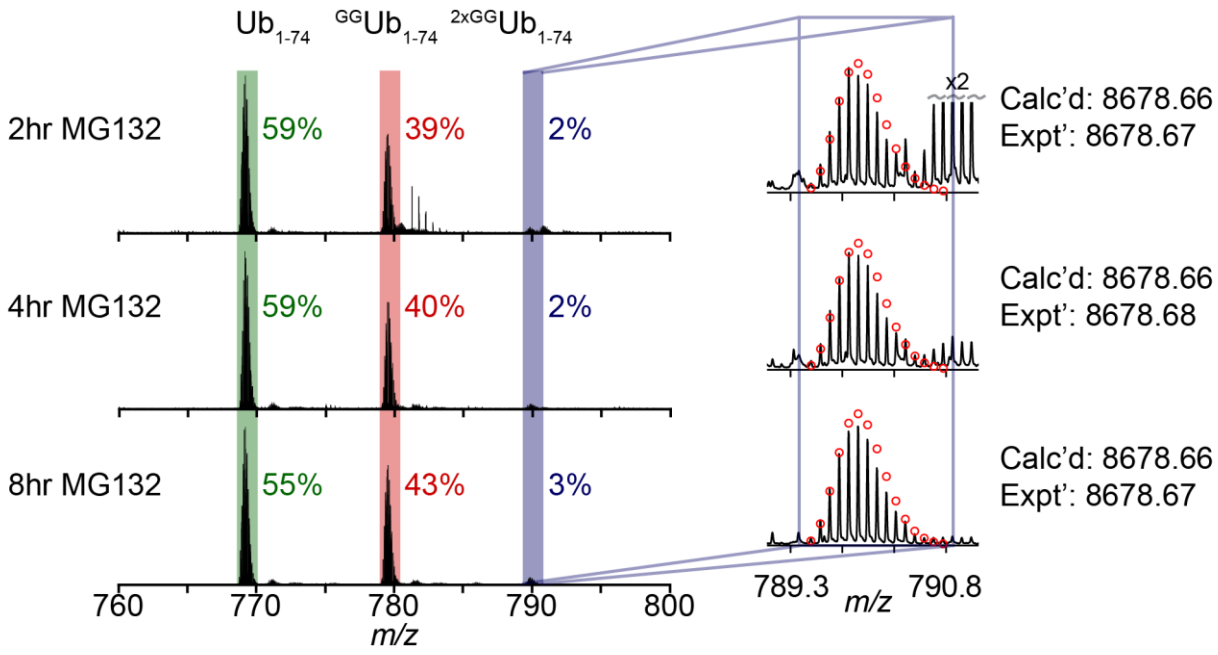


Figure S3: Representative MS1 data of the M^{11+} charge state for chains isolated from 2hr, 4hr, and 8hr MG132 treatments using agarose TUBEs. HEK cells were treated with 10 µM MG132 for the indicated times followed by UbiChEM-MS workflow using TUBE2. Represented here is the M^{11+} charge state for all three minimally digested Ub products - Ub_{1-74} (green box), $^{GG}Ub_{1-74}$ (red box), and $^{2xGG}Ub_{1-74}$ (blue box). The percentages represent the relative intensities calculated for all three Ub species. Calc'd - calculated most abundant weight; expt'l – experimental most abundant molecular weight.

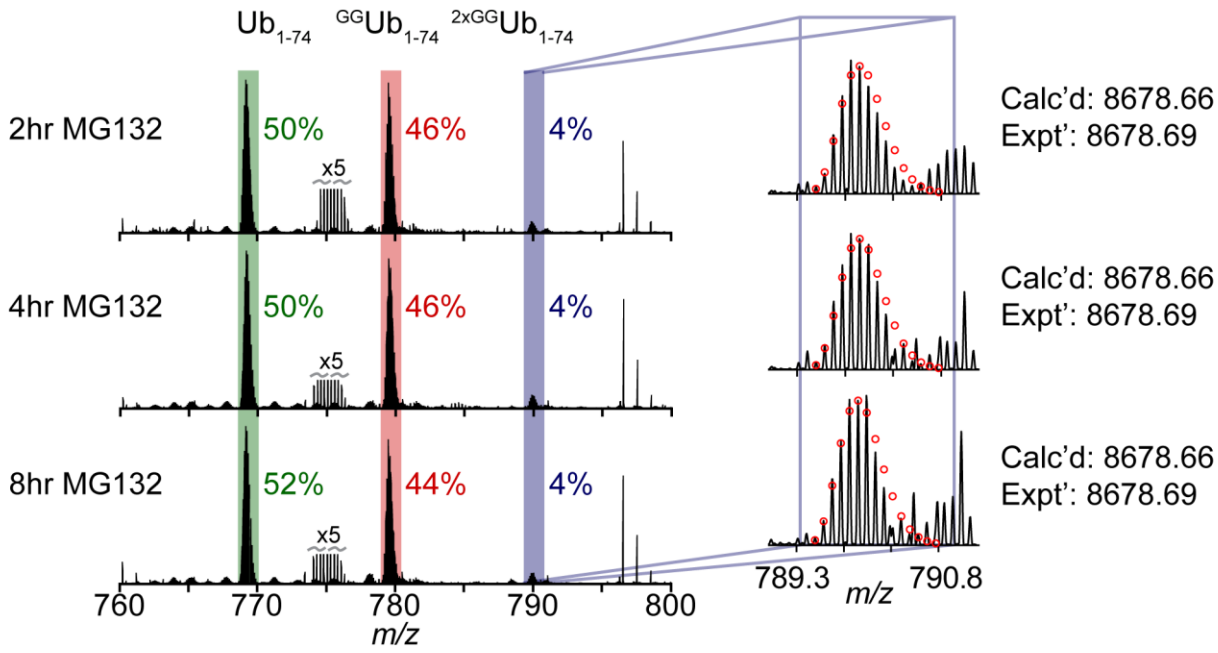


Figure S4: Representative MS1 data of the M^{11+} charge state for chains isolated from 2hr, 4hr, and 8hr MG132 treatments using Halo-NZF1. HEK cells were treated with 10 µM MG132 for the indicated times followed by UbiChEM-MS workflow using Halo-NZF1. Represented here is the M^{11+} charge state for all three minimally digested Ub products - Ub_{1-74} (green box), $^{GG}Ub_{1-74}$ (red box), and $^{2xGG}Ub_{1-74}$ (blue box). The percentages represent the relative intensities calculated for all three Ub species. Calc'd - calculated most abundant weight; expt'l – experimental most abundant molecular weight.

Table S2: Table of data used to make bar graph in Fig 2 for chains isolated with agarose TUBE2.

	Relative Abundance										
	Bio Rep1			Bio Rep 2			Bio Rep 3			Mean	SEM
	TR1	TR2	TR3	TR1	TR2	TR3	TR1	TR2	TR3		
Untreated											
Ub_{1-74}	56.0%	57.2%	57.2%	71.0%	70.6%	69.4%	45.5%	59.9%	59.6%	60.7%	2.8%
$^{GG}Ub_{1-74}$	42.7%	41.6%	41.6%	28.5%	28.8%	30.0%	51.9%	37.6%	38.3%	37.9%	2.6%
$^{2xGG}Ub_{1-74}$	1.3%	1.2%	1.2%	0.5%	0.5%	0.7%	2.7%	2.5%	2.2%	1.4%	0.3%
2hr MG132											
Ub_{1-74}	59.0%	58.8%	59.6%	66.0%	65.6%	67.7%	47.3%	47.2%	47.1%	57.6%	2.8%
$^{GG}Ub_{1-74}$	39.5%	39.7%	39.1%	33.2%	33.5%	31.3%	49.2%	49.5%	49.7%	40.5%	2.4%
$^{2xGG}Ub_{1-74}$	1.5%	1.5%	1.3%	0.8%	1.0%	1.0%	3.6%	3.3%	3.2%	1.9%	0.4%
4hr MG132											
Ub_{1-74}	58.0%	58.6%	58.3%	58.3%	58.3%	58.2%	48.0%	48.8%	47.9%	54.9%	1.7%
$^{GG}Ub_{1-74}$	40.9%	40.4%	40.7%	40.2%	40.2%	40.3%	50.8%	50.7%	50.8%	43.9%	1.7%
$^{2xGG}Ub_{1-74}$	1.1%	1.0%	1.1%	1.5%	1.5%	1.5%	1.2%	0.6%	1.3%	1.2%	0.1%
8hr MG132											
Ub_{1-74}	55.1%	58.1%	59.2%	51.6%	51.2%	48.4%	48.2%	48.1%	46.6%	51.8%	1.5%
$^{GG}Ub_{1-74}$	43.2%	40.3%	39.3%	45.4%	45.8%	49.9%	49.4%	49.5%	50.7%	45.9%	1.4%
$^{2xGG}Ub_{1-74}$	1.7%	1.5%	1.5%	3.0%	3.0%	1.8%	2.4%	2.4%	2.7%	2.2%	0.2%
16hr MG132											
Ub_{1-74}	45.3%	45.2%	45.0%	43.3%	43.1%	41.6%	43.7%	43.8%	44.3%	43.9%	0.4%
$^{GG}Ub_{1-74}$	51.2%	51.4%	51.6%	52.5%	52.9%	54.3%	52.8%	52.7%	52.1%	52.4%	0.3%
$^{2xGG}Ub_{1-74}$	3.5%	3.4%	3.4%	4.2%	4.0%	4.1%	3.5%	3.5%	3.6%	3.7%	0.1%

Bio Rep – Biological Replicates, TR – Technical Replicates, Mean – average across all biological and technical replicates, SEM – standard error of the mean

Table S3: Table of data used to make bar graph in Fig 3 for chains isolated with Halo-NZF1 domain.

	Relative Abundance				
	Bio Rep1	Bio Rep2	Bio Rep2	Mean	SEM
Untreated					
Ub1-74	54.3 %	47.9 %	53.7 %	51.9 %	2.1 %
Ub1-74 1xGG	41.1 %	49.0 %	42.0 %	44.0 %	2.5 %
Ub1-74 2xGG	4.6 %	3.1 %	4.3 %	4.0 %	0.45 %
2 hr MG132					
Ub1-74	49.7 %	52.3 %	52.1 %	51.4 %	0.83 %
Ub1-74 1xGG	46.5 %	44.7 %	44.9 %	45.4 %	0.56 %
Ub1-74 2xGG	3.8 %	3.0 %	3.1 %	3.3 %	0.26 %
4 hr MG132					
Ub1-74	49.9 %	52.6 %	52.5 %	51.7 %	0.89 %
Ub1-74 1xGG	45.7 %	43.9 %	44.0 %	44.5 %	0.59 %
Ub1-74 2xGG	4.4 %	3.5 %	3.5 %	3.8 %	0.30 %
8 hr MG132					
Ub1-74	52.6 %	53.9 %	54.1 %	53.5 %	0.49 %
Ub1-74 1xGG	44.1 %	43.4 %	43.1 %	43.5 %	0.28 %
Ub1-74 2xGG	3.4 %	2.7 %	2.7 %	2.9 %	0.22 %
16hr MG132					
Ub1-74	48.3 %	50.6 %	50.3 %	49.7 %	0.73 %
Ub1-74 1xGG	47.1 %	45.3 %	45.6 %	46.0 %	0.55 %
Ub1-74 2xGG	4.7 %	4.2 %	4.1 %	4.3 %	0.18 %

Bio Rep – Biological Replicates, Mean – average across all biological replicates, SEM – standard error of the mean

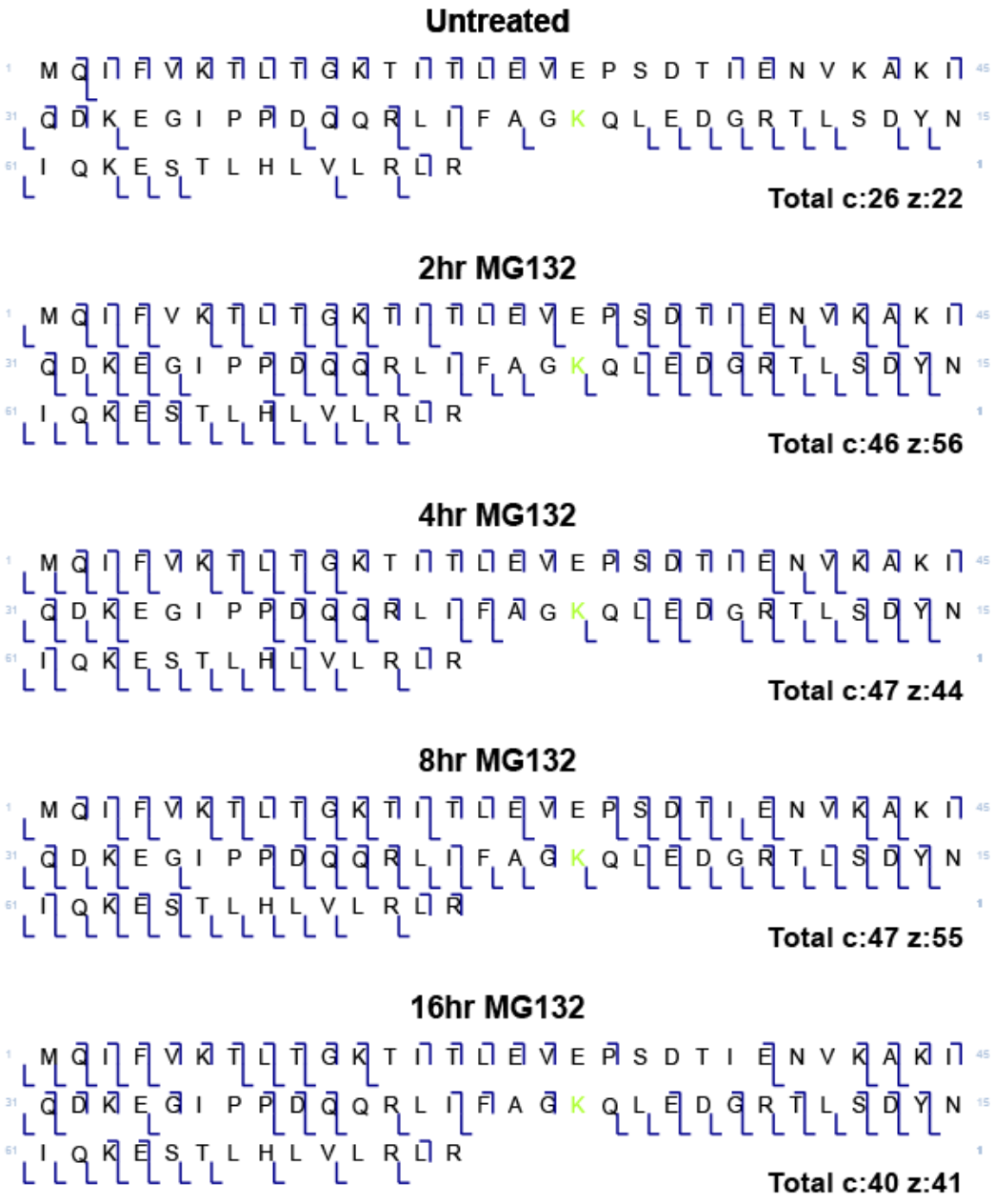


Figure S5. ETD analysis of $^{GG}Ub_{1-74}$ for chains isolated using agarose TUBEs. Map of ETD fragments that show $^{GG}Ub_{1-74}$ is primarily K48 linked Ub from untreated and MG132 treated samples.

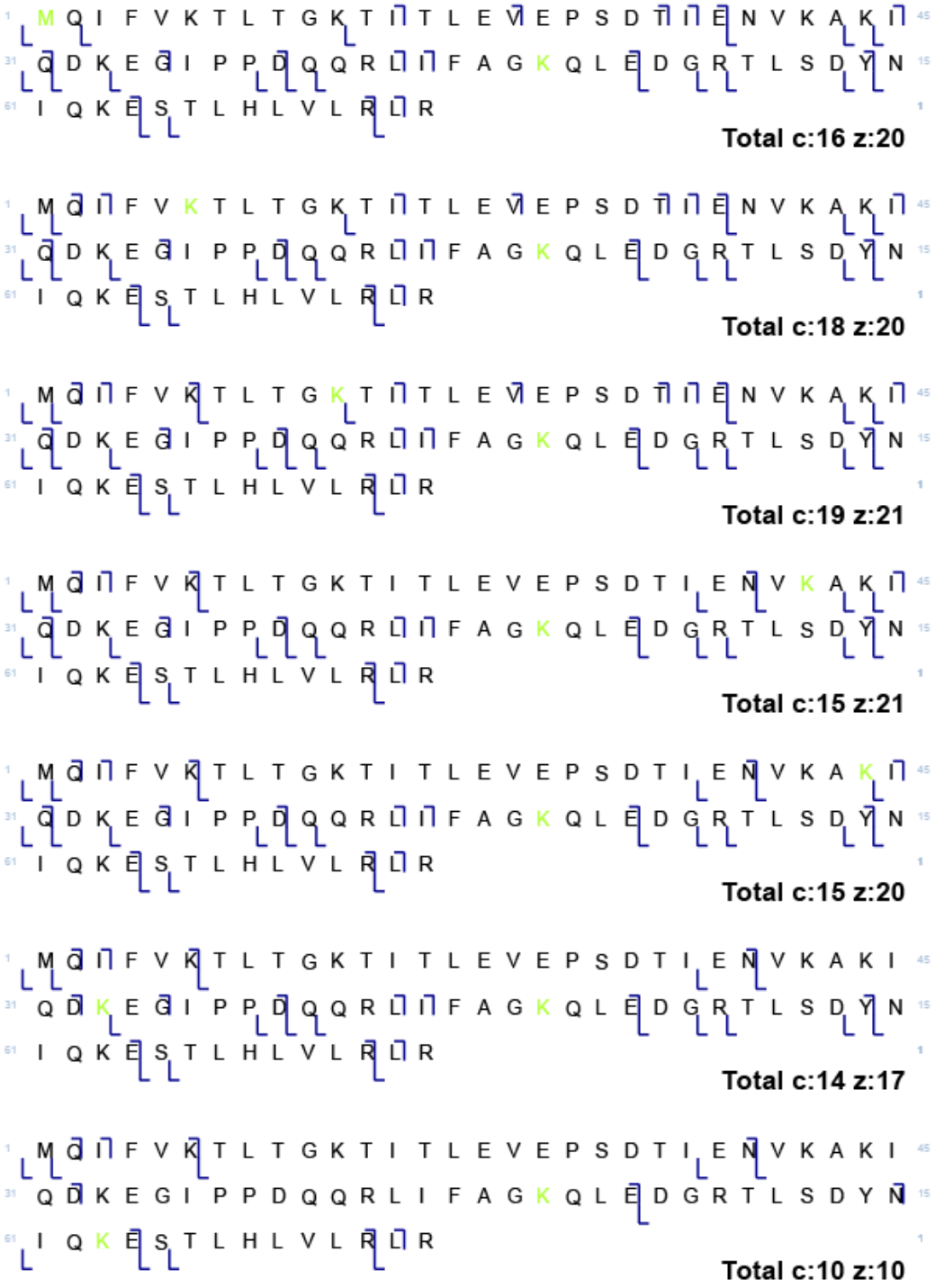


Figure S6. ETD analysis of $^{2xGG}Ub_{1-74}$ for chains isolated using agarose TUBEs. Map of ETD fragments showing that the absolute configuration for $^{2xGG}Ub_{1-74}$ cannot be unambiguously assigned, but is likely to contain a K48 linkage from untreated and MG132 treated samples.

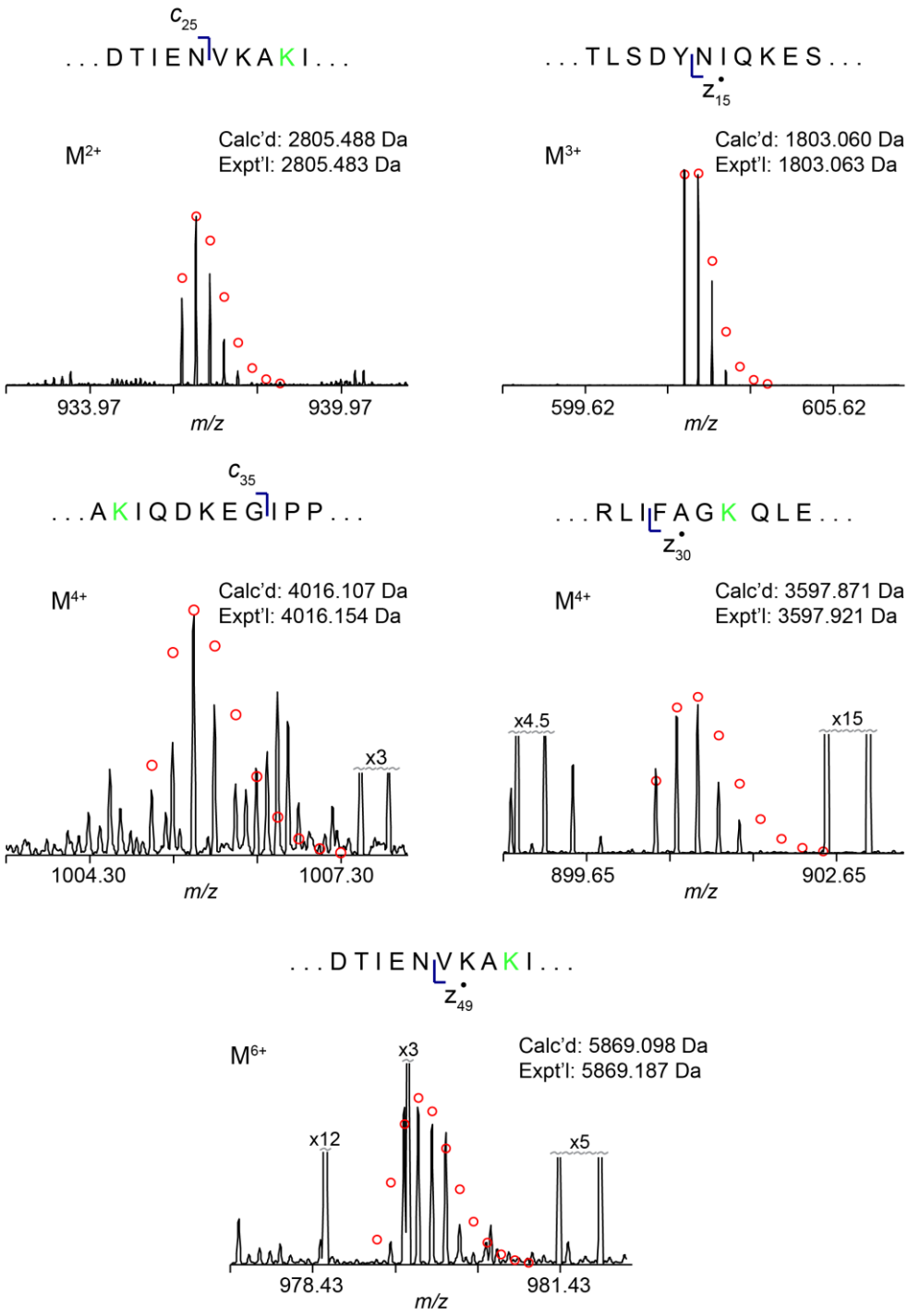


Figure S7. ETD fragments further supporting the modification of K29 and K48. ETD fragments that show the presence of modification on K29 and K48 as well as a lack of modification on M1, K6, K11, K33 and K63.

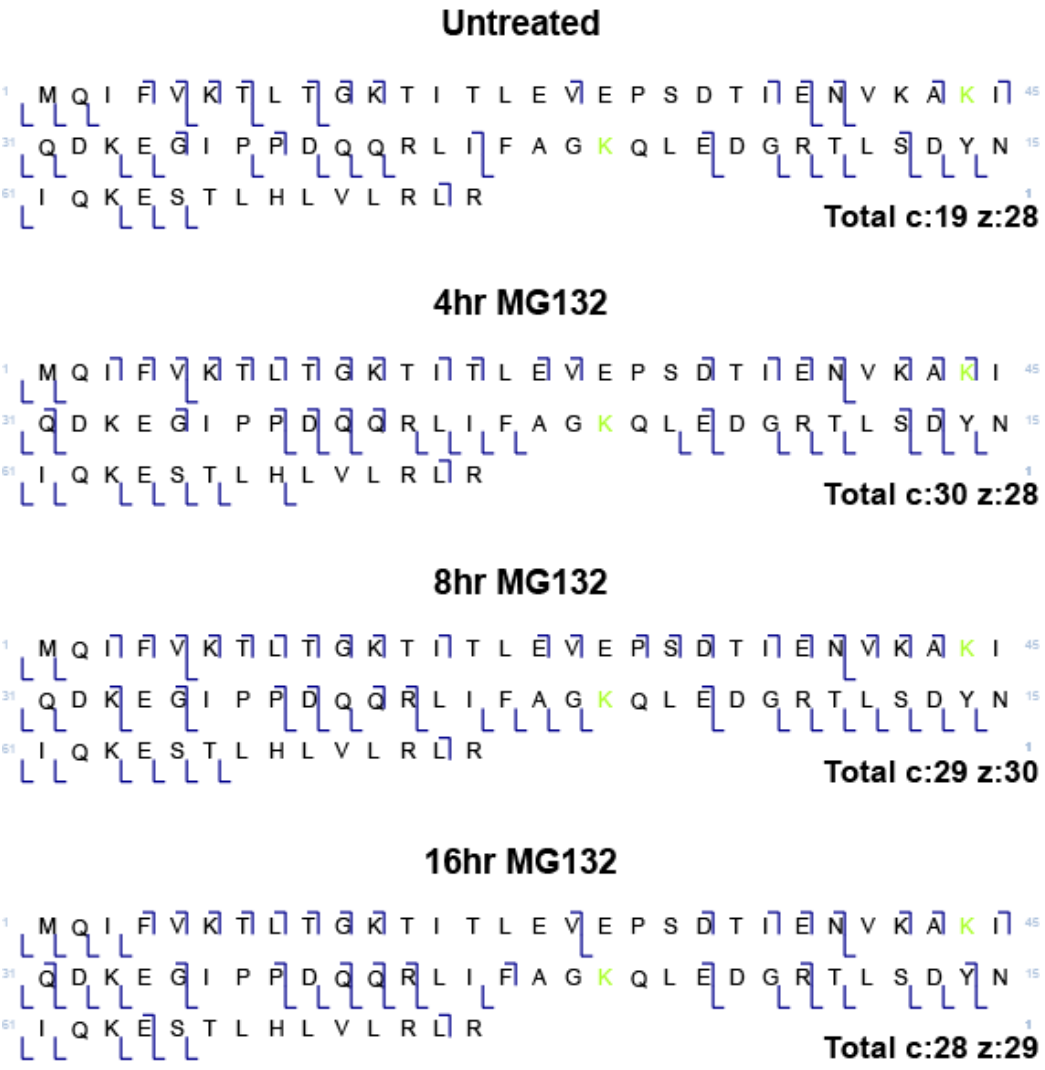


Figure S8. ETD analysis of $^{2xGG}Ub_{1-74}$ for chains isolated using Halo-NZF1. Map of ETD fragments assigning the $^{2xGG}Ub_{1-74}$ species contains a K29/K48 branched point from untreated and MG132 treated samples.

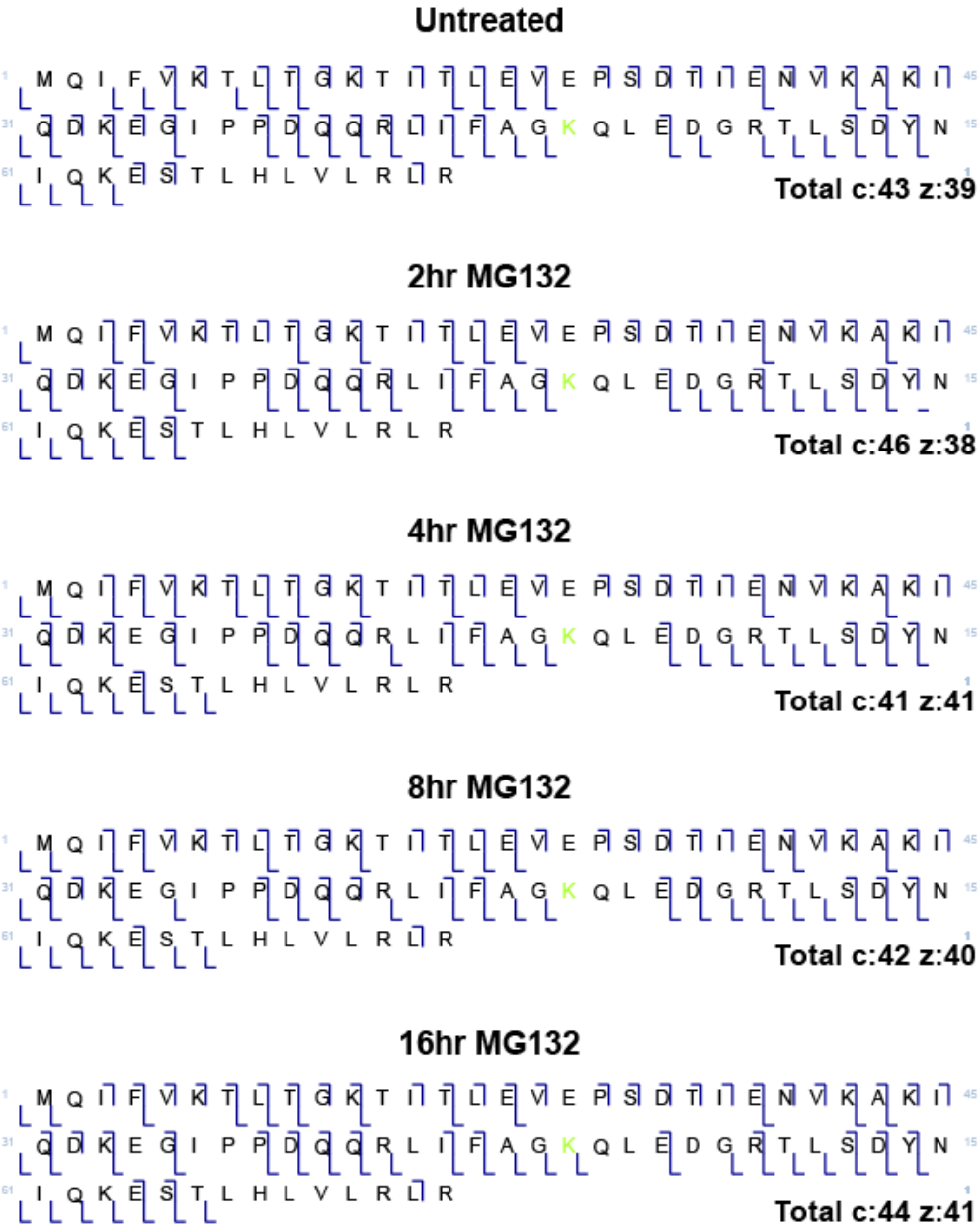


Figure S9. ETD analysis of $^{GG}Ub_{1-74}$ for chains isolated using Halo-NZF1. Map of ETD fragments that show that $^{GG}Ub_{1-74}$ is primarily K48 linked Ub from untreated and MG132 treated samples.

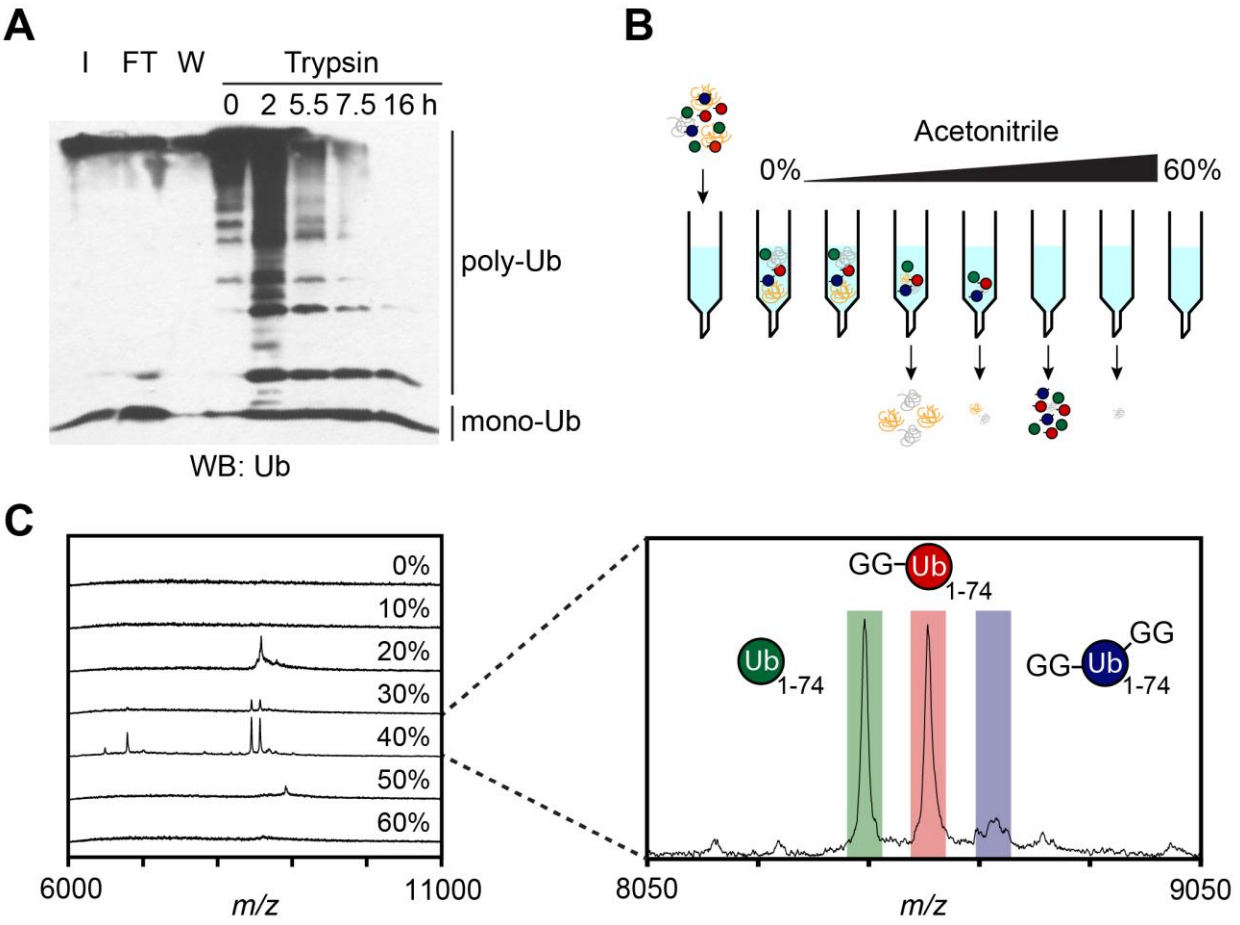


Figure S10. Optimization of middle-down MS for chains isolated using Halo-NZF1. A. Anti-Ub (P4D1) western blot showing the optimization of minimal tryptic conditions for chains isolated from untreated HEK cells. (*left to right*) Input lysate, Flow Through, Wash, Resin incubated with 2.5ug trypsin from 0 to 16hours. **B.** Cartoon depicting C_{18} column purification of minimally digested products. Acidified digests were applied to a 100mg Sep-Pak cartridge (Waters) and washed with an increasing gradient of acetonitrile. **C.** MALDI-TOF analysis of fractions from C_{18} column (*on left*). Ub species elutes in the 40% acetonitrile fraction. (*on right*) A zoomed-in view of 40% acetonitrile fraction showing all three products, Ub_{1-74} (*green box*), $^{GG}Ub_{1-74}$ (*red box*), and $^{2xGG}Ub_{1-74}$ (*blue box*).

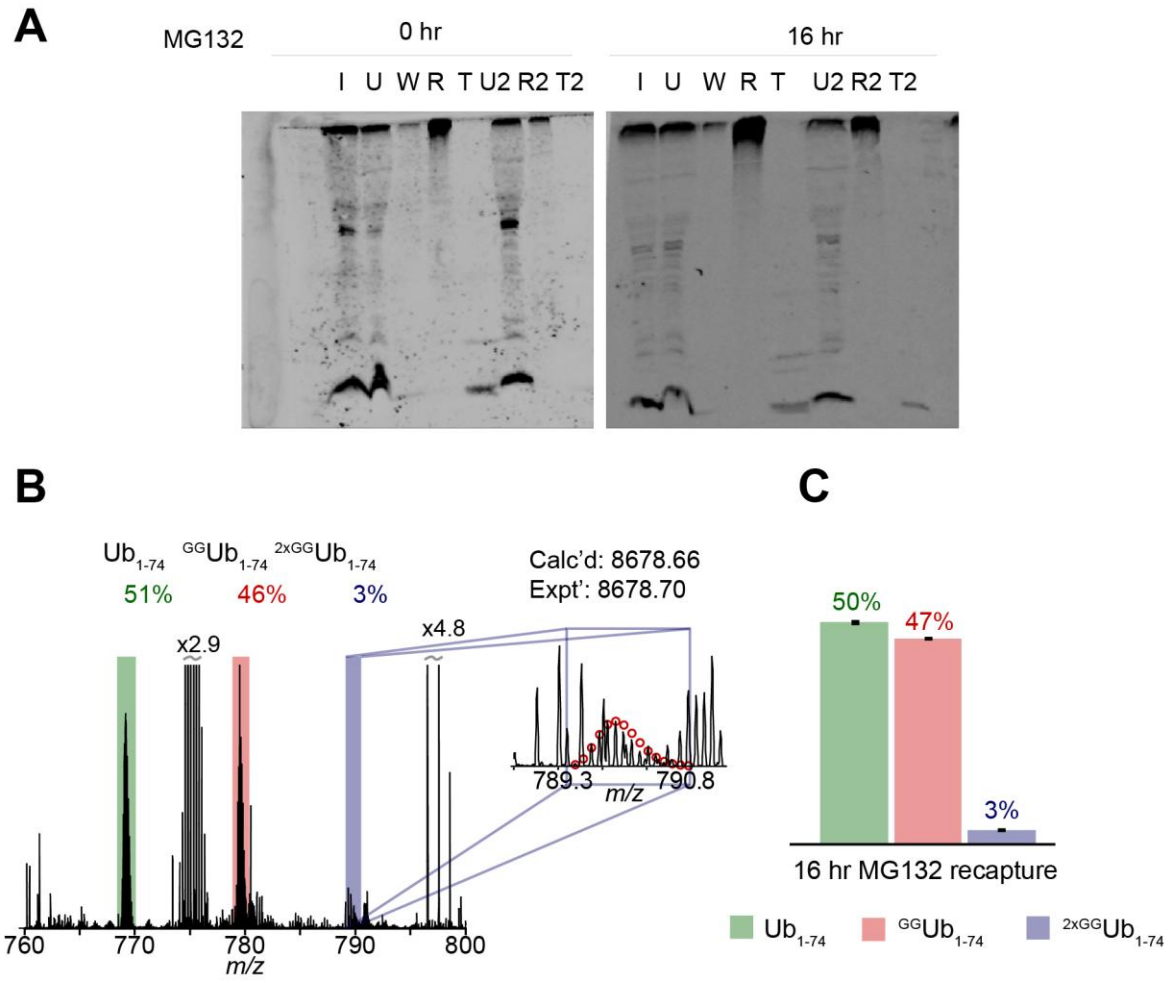


Figure S11. Results from Halo-NZF1 recapture experiment. **A.** Western blots analysis of recapture experiments. Ub chains were isolated using Halo-NZF1 by incubating 45 mg of HEK lysate with or without 16 hr MG132 treatment with 200 µL of the resin. The unbound lysate was further incubated with 200 µL of fresh resin and the fractions were analyzed by western blot. I = input, U = unbound, W = Wash, R = resin, T = minimal trypsinolysis, U2 = unbound after recapture, R2 = resin after recapture, T2 = minimal tryspinsolysis of recaptured material. **B.** The recaptured minimally trypsinized material from the 16 hr MG132 treatment was analyzed by ESI MS. The spectra shows the presence of all three minimally digested products - Ub_{1-74} (green box), $^{GG}Ub_{1-74}$ (red box), and $^{2xGG}Ub_{1-74}$ (blue box). The spectra correspond to the Ub^{11+} charge state for chains isolated from either untreated or MG132 treated HEK lysate. **C.** Quantification of Ub_{1-74} species recaptured by Halo-NZF1 resin. The percent distribution is calculated by averaging relative abundance of each Ub_{1-74} species to total abundance of all three species from three biological replicates for recapture. (Figure S4). Error bars represent SEM. Calc'd - calculated most abundant weight; expt'l – experimental most abundant molecular weight.

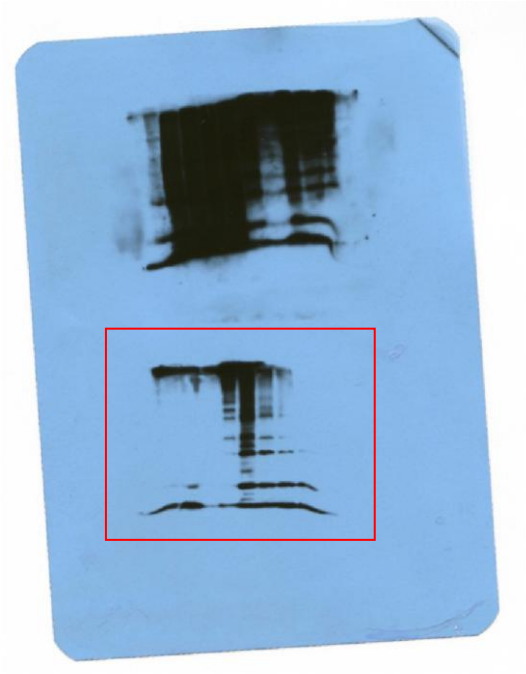


Figure S12. Full Western Blot used in Figure 2. Red box indicates the portion of data used in the figure.

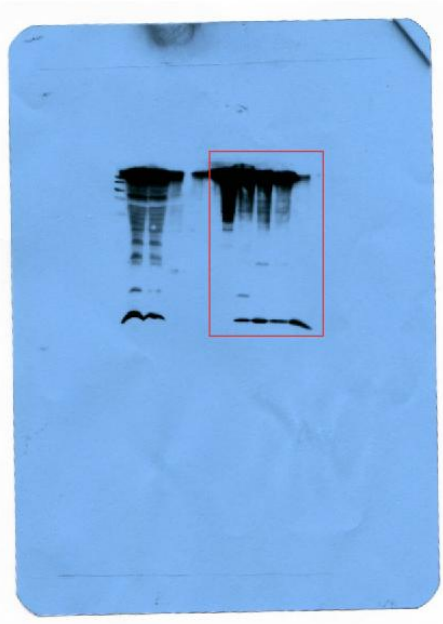


Figure S13. Full Western Blot used in Figure 4. Red box indicates the portion of data used in the figure.

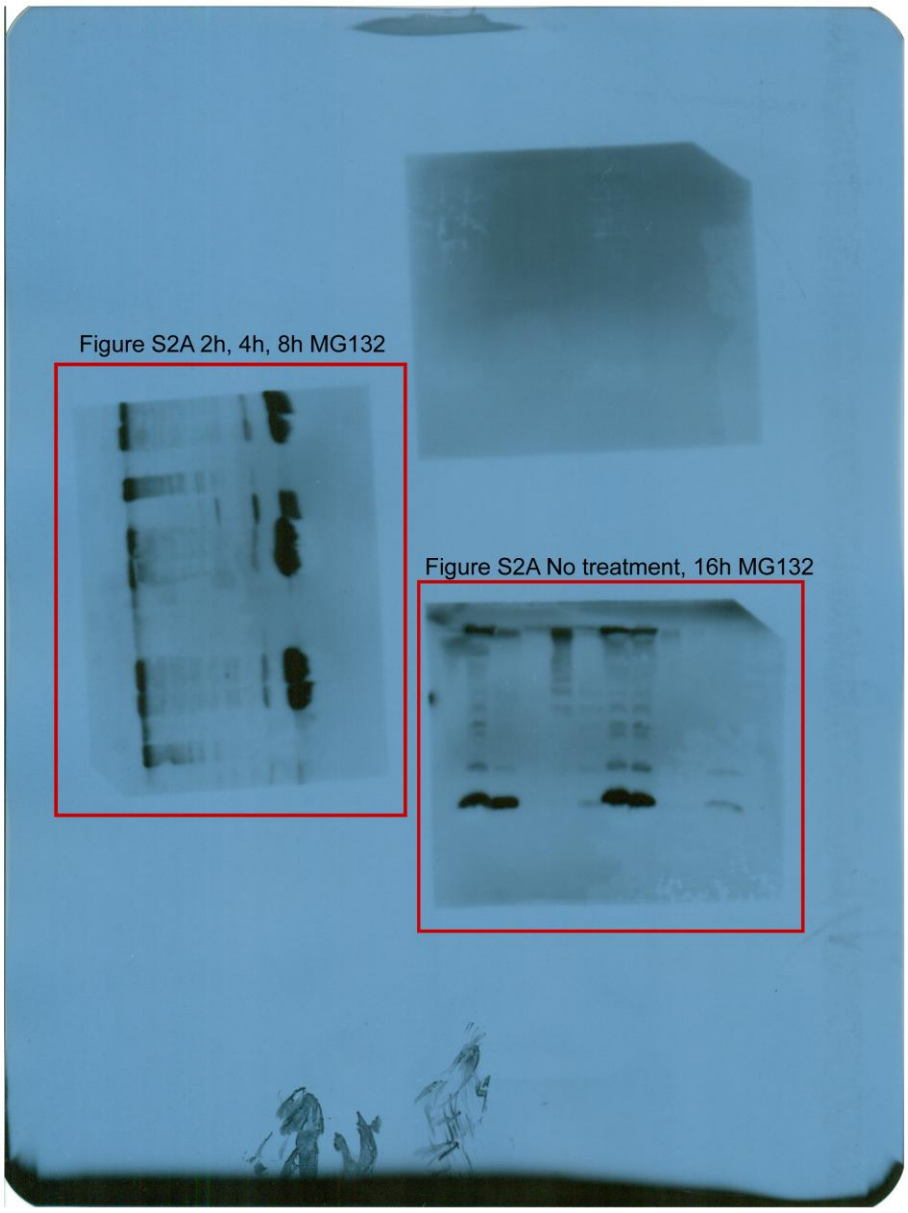


Figure S14. Full Western Blot used in Figure S2A. Red box indicates the portion of data used in the figure.

Figure S1

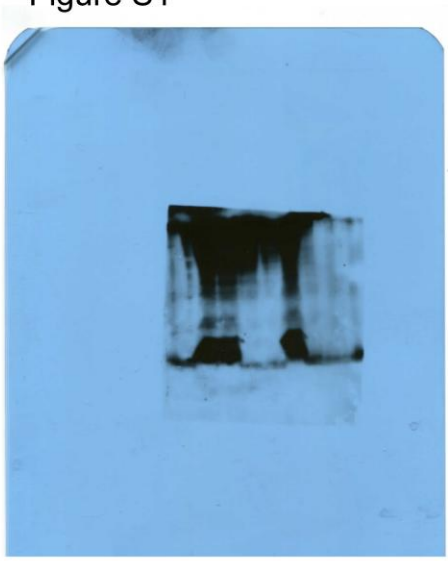


Figure S2B No treatment

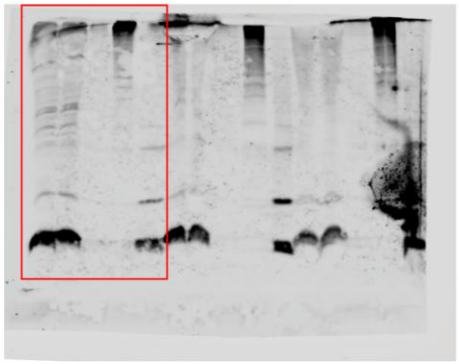


Figure S2B 16 hr MG132

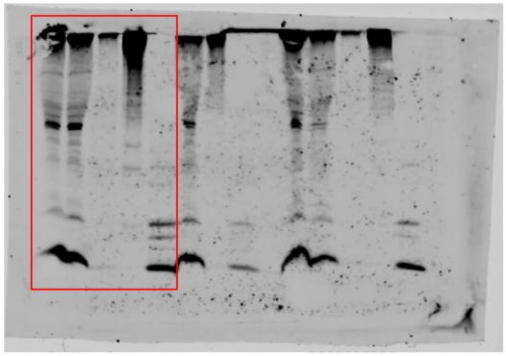


Figure S5 No treatment

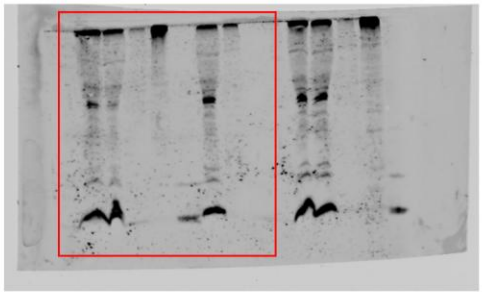


Figure S5 16 hr MG132

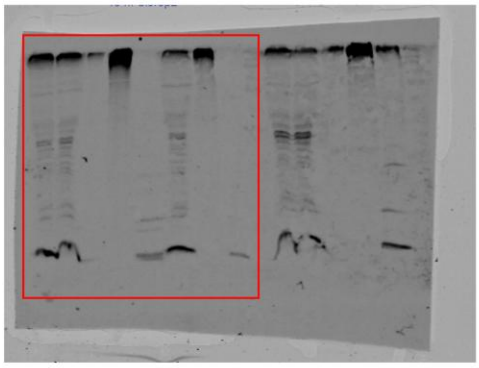


Figure S2B 2, 4, 8 hr MG132

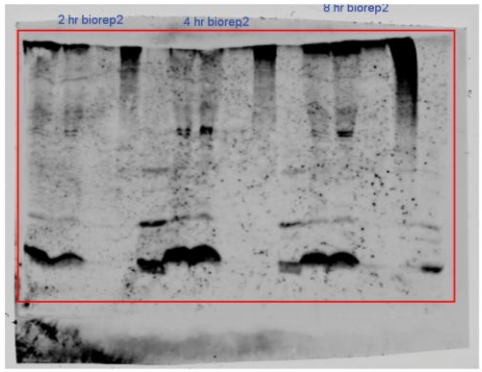


Figure S15. Full Western Blots used in Figures S1 and S2. Red box indicates portion of blot used in the figure when more than one blot appears on the same piece of film.

II. Materials

All reagents from commercial suppliers were – Agarose-TUBE2, purchased from LifeSensors (Cat. No. UM402); Halolink Resin, purchased from Promega (Cat. No. G1914); and MG132, purchased from Enzo Life Sciences (Cat. No. BML-PI102-0025). The antibodies used in this publication, ubiquitin monoclonal antibody (P4D1, Enzo Life Sciences, Cat. No. BML-PW0930-0100), Goat Anti-Mouse IgG Antibody, HRP conjugate (EMD Millipore Cat. No. 12-349), and IRDye® 800CW Goat anti-mouse IgG (Li-Cor Cat. No. 925-32210).

III. Protein Expression and Purification

a. Expression and purification of His_6-MBP-Halo- NZF1 fusion protein.

The Halo-NZF1 (aa1-33 of TRABID) fusion protein was cloned into the pDB.His.MBP vector between the NdeI and NotI restriction sites and purified using a method similar to the one described previously.[1] The fusion protein was expressed in *E. coli* Rosetta (DE3) in LB media supplemented with kanamycin (50µg/mL) and 200 µM $ZnSO_4$. Cells were grown to OD_{600}~0.6 at 37^{o}C and induced with 300µM IPTG. Upon induction, the temperature was lowered to 16^{o}C and protein expressed overnight for 16hours. The cells were centrifuged at 5000 *xg* for 20mins at 4^{o}C and the cell pellet can be stored at -80^{o}C until purification. This cell pellet was resuspended in 40mL lysis buffer (50mM Tris pH 7.5, 300mM NaCl, 0.5mM TCEP, 1mM PMSF, and 1mM benzamidine) and lysed by sonication. The resulting lysate was then clarified at 31,000 x*g* for 45mins at 4^{o}C and the protein was isolated by Ni-NTA chromatography. Fractions containing the MBP-Halo-NZF1 fusion protein were then concentrated and buffer exchanged into a low salt buffer (50mM Tris pH 7.5, 50mM NaCl). TEV protease was then added to remove the MBP tag, and cleavage was allowed to proceed overnight at 4^{o}C. The cleavage mixture was then poured over Ni-NTA resin to remove 6xHis-MBP as well as any uncleaved fusion protein. The resulting protein was then further purified by ion exchange chromatography (MonoQ, GE Healthcare). Fractions containing the desired protein – Halo-NZF1, were then exchanged into storage buffer (50mM Tris pH 7.5, 50mM NaCl, and 10% glycerol) and stored at -80^{o}C.

b. Expression and purification of His_6-MBP-OTUB1.

OTUB1 was expressed recombinantly in BL21 (DE3) *E. coli.*, with an N-terminal 6xHis-MBP (maltose binding protein) tag. Cells were grown in LB media at 37^{o}C with shaking at 180rpm until OD_{600}~0.6 and subsequently expression was induced with IPTG (100µM). Upon induction, the temperature was lowered to 16^{o}C and protein expressed overnight for 16hours. The cells were centrifuged at 5000 x*g* for 20mins at 4^{o}C. This cell pellet was resuspended in cold lysis buffer (20mM Tris HCl pH7.8, 4mM $MgCl_2$, 100mM NaCl, 1mM TCEP, and 0.25mM PMSF) and lysed by sonication. The resulting lysate was clarified at 75,000 x*g* at 4^{o}C and His6-MBP-OTUB1 was isolated by cobalt affinity chromatography. Fractions containing the fusion protein were buffer exchanged into TEV protease buffer (20mM Tris pH 8.5, 150mM NaCl, 2.5mM NaCl, 2mM DTT) and treated with TEV protease overnight at 4^{o}C to remove the MBP tag. The protein was then buffer exchanged into MonoQ buffer A (20mM Tris pH 7.4, 1mM DTT) and purified by ion exchange chromatography (MonoQ, GE Healthcare) running a gradient of 0 to 50% buffer B (20mM Tris pH 7.4, 1M NaCl, 1mM DTT) over 20 column volumes. Fractions containing OTUB1 were identified by SDS-PAGE, buffer exchanged into storage buffer (50mM HEPES pH 8.0, 50mM NaCl, 2mM DTT and 10% glycerol) and flash frozen and stored at -80^{o}C.

c. Expression and purification of His6-MBP-USP15.

USP15 was expressed recombinantly in BL21 (DE3) *E. coli*., with an N-terminal 6xHis-MBP tag. Cells were grown at 37ºC with shaking at 180rpm until OD_{600}~0.4. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.2mM). Cells were then grown

overnight at 16ºC and then harvested by centrifugation for 30mins at 4000 x*g*, 4ºC. The cell pellet was resuspended in lysis buffer (50mM Tris pH 7.5, 100mM NaCl, 1mM TCEP, and 0.25mM PMSF) and then lysed by sonication. The lysate was clarified by centrifugation at 75000 x*g* for 30mins at 4ºC. USP15 was then purified using two chromatographic steps: nickel affinity chromatography followed by anion exchange after the MBP fusion was removed using TEV protease.

d. Expression and purification of His_6-GST-OTU+AnkUBD TRABID.

The pOPINK-TRABID plasmid was purchased from addgene and purified as described previously.[2] OTU+AnkUBD TRABID (aa245-697) was expressed in Rosetta 2(DE3) cells with an N-terminal 6XHis-GST tag. Cells were grown in LB media to an OD_{600} ~0.8-1.0 at 37°C and induced with 1mM IPTG. Upon induction the temperature was lowered to 20°C, protein expressed for 18-20 hr and then the cells were pelleted at 5000 x*g* for 20 minutes at 4°C. The cell pellet was resuspended in cold lysis buffer (25mM Tris pH 8.5, 200mM NaCl, 5mM DTT) and lysed by sonication. The lysate was then clarified by centrifugation at 30000 x*g* for 45min. The clarified lysate was incubated in 2mL pre-equilibrated GST resin for 1.5hr and washed with 25mM Tris pH 8.5, 100mM NaCl, 5mM DTT. The GST tag was cleaved from OTU-AnkUBD TRABID on resin with 50U GST-tagged HRV 3C Protease (Fisher Scientific) overnight at 4°C. Cleaved protein was buffer exchanged into Buffer A (25mM Tris pH 8.5, 5mM DTT) and further purified using anion exchange chromatography (MonoQ, GE healthcare) running a gradient of 0 to 50% buffer B (25mM Tris pH 8.5, 500mM NaCl, 5mM DTT). OTU+AnkUBD TRABID fractions were identified by SDS-PAGE, buffer exchanged into storage buffer (50mM Tris pH 7.5, 75mM NaCl, 2mM DTT, and 10% glycerol), concentrated and flash-frozen.

IV. Cell Culture, Treatment and Lysis

HEK293 cells were grown in high glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1x Glutamax (Life Technologies Inc.), and antibiotics (100U/mL penicillin, and 100mg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2. Once cells reached 80-90% confluency, they were washed with sterile PBS (pre-warmed to 37°C), and then grown in media containing inhibitor 10µM MG132 at 37°C. After four hours, cells were harvested in 2mL cold PBS for every 150mm plate used. Cells were pelleted at 800 x*g* for two minutes and the supernatant decanted. Cell pellets were washed two more times with cold PBS, resuspension and centrifugation at 800 x*g* as previous step.. For every 150mm plate of HEK cells, 1mL of cold lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 0.05% IGEPAL® - CA-630 (SIGMA), 10mM 2-chloroacetamide, 10mM N-ethylmaleamide, and 1x protease inhibitor cocktail (Gold Biotechnology)) was added. Cells were incubated in lysis buffer on ice for 30mins prior to sonication and then the resulting lysates were clarified at 16,000 x*g* and 4°C for 30mins.

V. Enzymatic assays

a. Time course analysis of minimal tryptic digests

Halo-NZF1 resin (200µL) containing Ub chains isolated from 63.4mg of untreated input lysate were resuspended in 100µL of minimal buffer. Sequencing-grade modified trypsin (2.5µg, Promega) was then added to the suspension and the proteolysis was allowed to proceed at room temperature up to 16hours. Aliquots (20µL) of the slurry were then quenched by the addition of 6x Laemmli loading buffer at the indicated time points. The quenched reaction mixtures were then separated on a 15% SDS-PAGE gel and analyzed by western blot with anti-Ub (P4D1). Table S1 contains empirically determined optimal cell lysate to trypsin ratios for different cellular treatments used in this study.

b. DUB assay analysis of Isolated Ub chain linkages

MG132 treated HEK cell lysate (22.5mg) was incubated with Halo-NZF1 resin (100uL) overnight at 4^{o}C with shaking/stirring. The resin was then washed with 1mL of binding buffer four times followed by 2x washes with 1 mL DUB buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM DTT) and then the resin was resuspended as a 50% slurry in DUB buffer. The resin was aliquoted into four 20µL portions (40µL of the 50% slurry) and warmed to 37^{o}C for 3mins. After warming, either TRABID (1.7µM final concentration), OTUB1 (3.3µM final concentration), both TRABID and OTUB1 (1.7µM TRABID and 3.3µM OTUB1), or USP15 (1.7µM) were added and the resulting mixtures were rotated at 37^{o}C overnight. The reactions were then quenched by the addition of 6x Laemmli loading dye. The samples were then separated on a 15% SDS-PAGE gel and analyzed by western blot using anti-Ub antibody (P4D1).

VI. Supplemental References

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