Supplemental Information

Triubiquitin probes for identification of reader and eraser proteins of branched polyubiquitin chains

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Supplemental Methods

Purification of Ub species. HA-Ub_{1-75}-MESNA was generated and purified as previously described[1]. A HA-Ub_{1-75} intein fusion gene in pTYB1 plasmid was transformed into E. coli BL21(DE3) cells. LB media supplemented with 100 μg/mL ampicillin was used to culture the cells at 37 °C. At OD_{600} of 0.6, cell culture was induced with 0.4 mM IPTG and cultured further for 16–18 hrs at 16 °C. Harvested cells by centrifugation were resuspended in a 20 mM Tris (pH 7.5) buffer containing 200 mM NaCl, 1 mM EDTA, 5% glycerol and lysed on ice via sonication. The cell supernatant was incubated with chitin resin (NEB) at 4 °C overnight or r.t for 2 hrs. Following wash with 20 mM Tris (pH 7.5), 1 M NaCl, 1 mM EDTA, and 5% glycerol, the chitin resin was equilibrated with 20 mM MES (pH 6.5), 100 mM NaCl. The resin was then incubated with 50 mL cleavage buffer containing 20 mM MES (pH 6.5), 100 mM NaCl, 75 mM MESNA for 12 hrs at r.t. and eluted to yield Ub_{1-75}-MESNA. Further purification was performed on a HiLoad Superdex 200 pg 16/60 column (GE Healthcare). The double Lys-to-Cys mutant Ub_{1-75}-MESNA was also prepared following a similar procedure.

K63R Ub_{1-76}/pET3a, K48R Ub_{1-76}/pET3a, K11C/D77 Ub/pET3a, and K63C/D77 Ub/pET3a in BL21(DE3) *E. coli* cells were grown in LB media in the presence of 100 μg/mL ampicillin at 37 °C until OD_{600} reached 0.6, then induced with 0.4 mM IPTG and grown at 17 °C for 16 hrs. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4 °C, and resuspended in a lysis buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM PMSF, 5 % Glycerol, 1 mM EDTA). The cell lysate was centrifuged at 14,000 rpm for 30 minutes at 4 °C to remove the cellular debris. The supernatant was subjected to heat inactivation at 95 °C for 15 minutes, then centrifuged at 14,000 rpm for 30 minutes. The insoluble fraction was discarded, and the heat treatment was repeated. To the final supernatant, 80% ammonium sulfate was added at 4 °C followed by stirring for 10 minutes before centrifugation at 14,000 rpm for 20 minutes to collect the pellet containing the precipitated ubiquitin. The pellet was resuspended in a gel filtration buffer (50 mM Tris, pH 7.5, 150 mM NaCl), syringe filtered, and then purified using a Superdex 75 10/300 gel filtration column. The resulting protein was concentrated and confirmed by mass spectrometry.

Purification of tUIM domains. Genes for UIMC1 (a.a. 76-128), USP37 (a.a. 700-851) and AN13A (a.a. 479-594) were synthesized and cloned into pET28a-HIS-TEV vector by Epoch Life Sciences, Inc. The proteins containing UIM domains were expressed and purified from transformed BL21(DE3) cells. The cells were grown at 37 °C until OD_{600} of 0.6, then induced with 0.4 mM IPTG and grown at 17 °C for 16 hrs. The cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in a lysis buffer containing 25 mM Tris (pH 8.0), 250 mM NaCl, 2 mM ß-ME, 1 mM PMSF for 30 minutes at 4 °C. The cells were lysed and the lysate was centrifuged at 14,000 rpm for 30 minutes at 4 °C to remove the cellular debris. The cell lysate containing the His-tagged UIM was incubated with Ni-NTA resin for 2 hrs rotating at 4 °C, then washed with 20 CV of buffer containing 10 mM imidazole and eluted from the resin with 2 CV buffer containing 250 mM imidazole. The UIM-containing protein was then purified with a HiTrap Q FF anion exchange column (Buffer A: 50 mM Tris, pH 8.0, 1 mM DTT; Buffer B: 50 mM Tris, pH 8.0, 1 M NaCl, 1 mM DTT). The protein was further purified on a Superdex 75 10/300 size exclusion chromatography column in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT.

Purification of deubiquitinases. The pOPINK-OTUD2 (full-length, a.a. 1-348, Addgene plasmid # 61409) and pOPINK-OTUD3 (OTU+UBA, a.a. 52-275, Addgene plasmid # 61411) plasmids were gifts from David Komander[2]. They were used for expression of His-GST-OTUD2-FL and His-GST-OTUD3 (OTU+UBA domains). Cells were

cultured, harvested and lysed in lysis buffer (25 mM Tris, pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM β-mercaptoethanol) cooled to 4 °C. Lysate was then centrifuged at 36,000 ×*g* for 30 min at 4 °C. The protein was batch absorbed using Ni-NTA resin (Qiagen), washed with 25 mM Tris (pH 8.0), 500 mM NaCl, 25 mM imidazole and eluted with an elution buffer (25 mM Tris, pH 8.0, 250 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol). The eluted fractions were combined, concentrated and buffer exchanged to a buffer containing 25 mM Tris (pH 8.0), 50 mM NaCl, 1 mM DTT and further purified by an anion exchange chromatography using a HiTrap Q FF column at 4 °C. Pure fractions were collected and concentrated. The protein purity was analyzed by SDS-PAGE.

Generation of diUb with native linkages. 5 mg/mL of monoubiquitin species were used in conjunction with 0.1 µM E1 and 20 µM of the respective E2 ubiquitin-conjugating enzymes overnight at 37 °C in a buffer containing 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 U/mL inorganic pyrophosphatase, 0.6 U/mL creatine phosphokinase, 0.1 mM DTT and 0.5 mM ATP to generate native diubiquitin. K63R Ub was reacted with D77 or K11C/D77 Ub using mouse E1 (mE1) and Mms2/Ubc13 to make K63 diubiquitin (K63-diUb) or K63 diUb containing a free Cys at K11 of the proximal ubiquitin. K48R Ub was reacted with D77 Ub, K11C/D77 Ub or K63C/D77 Ub using mE1 and E2-25K to generate K48-linked diUb or diUbs containing a free Cys replacing either K11 or K63 in the proximal ubiquitin. K11C Ub was reacted with D77 Ub using mE1 and UBE2S-UBD followed by AMSH treatment to generate K11-linked diubiquitin (K11-diUb)[3]. The diubiquitins were purified using a cation exchange HiTrap SP FF column (Buffer A: 50 mM ammonium acetate, pH 4.5, 1 mM DTT; Buffer B: 50 mM ammonium acetate, pH 4.5, 1 M NaCl, 1 mM DTT).

Generation of diUb-NC1-PA2 and diUb-NC1. DiUb-NC1-PA2 and diUb-NC1 were prepared following a previously published method[4]. To make diUb-NC1-PA2 probes, HA-$Ub_{1–75}$-MESNA was reacted with the noncleavable linker (NC) molecule and deprotected as previously described to obtain HA-$Ub_{1–75}$-NC[5]. In parallel, a mutant $Ub_{1–75}$-MESNA with a Lys-to-Cys mutation at the selected lysine site was reacted with propargylamine (PA) as described above to generate Cys-Ub-PA followed by a cation-exchange HiTrap SP FF column purification. Equal molar of HA-Ub-NC and Cys-Ub-PA were incubated at 0.5 mg/mL each in a 20 mM MES (pH 6.5) buffer containing 0.03 mM EDTA for 1 h at room temperature. Subsequently, the reaction mixture was purified using a cation exchange HiTrap SP FF column followed by purification using a Superdex 75 10/300 size exclusion column in 20 mM MES (pH 6.5) buffer to yield the diUb-NC1-PA2 probes. K63-diUb-NC1 was prepared similarly by reacting equal molar $Ub_{1–75}$-NC with K63C Ub, further purified using a cation exchange HiTrap SP FF column.

Culture and generation of HEK-293T cell lysates. Human HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 in a 75 mm^3 T-flask in a humidified incubator. To generate cell lysates, adhered cells were washed with cold DPBS buffer and harvested using 0.25% trypsin. The cells were pelleted and washed three times with additional DPBS buffer and resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 1 mM PMSF, 2 mM ATP, 0.5% Triton X-100 and 10% glycerol). The resuspension was sonicated to lyse the cells and the cell lysate was centrifuged for 15 min at 14,000 rpm at 4 °C and supernatant stored at -80 °C until further use.

Proteomic data analysis using MaxQuant and Perseus. Raw files (three replicates for each probe and bead control pulldown) were analyzed together using MaxQuant software version 1.4.0.6 as previously described[6]. In summary, peak list was searched with built-in Andromeda search engine against the UniProt human database downloaded from Uniprot website. MaxQuant parameters were set as follow unless otherwise indicated.

Multiplicity was set to one (no isotope labelling), and trypsin was set to cleave after lysine and arginine, unless followed by a proline. Maximum two missed cleavages were allowed, and maximum charge of peptide was set to 7. Seven amino acids were set to be the minimum peptide length. Carbamidomethylation of cysteine was a fixed modification. N-terminal acetylation, methionine oxidation, phosphorylation (serine, threonine, and tyrosine), and ubiquitin diglycine remnant were set as variable modifications. Label-free Quantification (LFQ) module was enabled with a minimum ratio count of 1, and second peptide search and re-quantify functions were enabled. The maximum number of modifications was set to 5 per peptide. The option of requiring MS/MS for LFQ comparisons was enabled. Target decoy approach was used to filter peptide spectrum matches (PSM), and a false discovery rate (FDR) of 1% was used for protein identification. The output text file "protein groups" was imported and analyzed using Perseus (software version 1.6.1.3). Proteins categorized as "only identified by modified peptides" were eliminated. Proteins identified with reversed decoy mode and the common contaminant proteins were excluded. Experimental replicates (3 total) were grouped. Proteins with at least one valid LFQ values (LFQ intensity >0) in at least one group were kept. Histogram of pre-imputed LFQ values showed relatively normal distribution which was used to impute missing values. Missing values were imputed using the normal distribution of the total matrix using the default parameters (0.3 width and 1.8 down shift) in Perseus.

Using Perseus multi-sample ANOVA test was performed with FDR of 0.01 and S0 = 1. ANOVA enriched Log_2(LFQ) values were transformed using Z-score normalization for each protein group and averaged for control and each probe pulldown. Heatmap was generated using hierarchical clustering with complete linkage method. Spiderplots were generated using Excel from the bead subtracted log_2(LFQ) values for the probes. Gene ontology (GO) analysis was performed on clusters using online (www.geneontology.org) and domain batch analysis was performed using SMART database (www.smart.embl.de) and visualized using iTol (www.itol.embl.de). Sequence alignments of relevant proteins were performed with Clustal Omega (www.ebi.ac.ud/Tools/msa/clustalo/).

Labelling of recombinant DUBs using mono-, di-and triUb-PA probes. Labelling of DUBs with the activity-based Ub probes (ABPs) was performed in 20 mM Tris (pH 8.0), 50 mM NaCl and 1 mM DTT at r.t. for 2 hrs with 1 µM probes and 2 µM enzymes. The reaction was quenched with 6X loading dye with 10 mM DTT and loaded onto an SDS-PAGE gel. The gels were fixed and stained with Coomassie brilliant blue, destained and visualized.

Western blotting analysis of lysate labelling by di-and tri-Ub-PA probes. 50 µg HEK-293T cell lysate and 1 µM HA-tagged monoUb, diUb and triUb probes were incubated in the lysis buffer to a final volume of 10 µL for 2 hrs at room temperature. The labelling reaction was quenched using 6X gel loading dye (35 mM Tris, 10% SDS, 30% glycerol and 0.81 M DTT, pH 6.8) and heated on a heat block at 95 °C for 10 min. Following centrifugation, sample was loaded and resolved on a 12% SDS-PAGE gel followed by transfer onto a PDVF membrane (Thermo Fisher Scientific Inc). The membrane was cut below the 50 kDa ladder band and blocked with 5% skim milk. The top half membrane was blotted with mouse anti-HA antibody in 5% BSA followed by a secondary HRP-conjugated anti-mouse antibody. The bottom half membrane was immunoblotted for GAPDH loading control with a mouse anti-GAPDH antibody in 5% BSA followed by blotting with a secondary HRP-conjugated anti-mouse antibody. Membranes were incubated with ECL Western blotting substrate (Thermo Fisher Scientific Inc., Waltham, MA) and signals were imaged.

Supplemental Figures

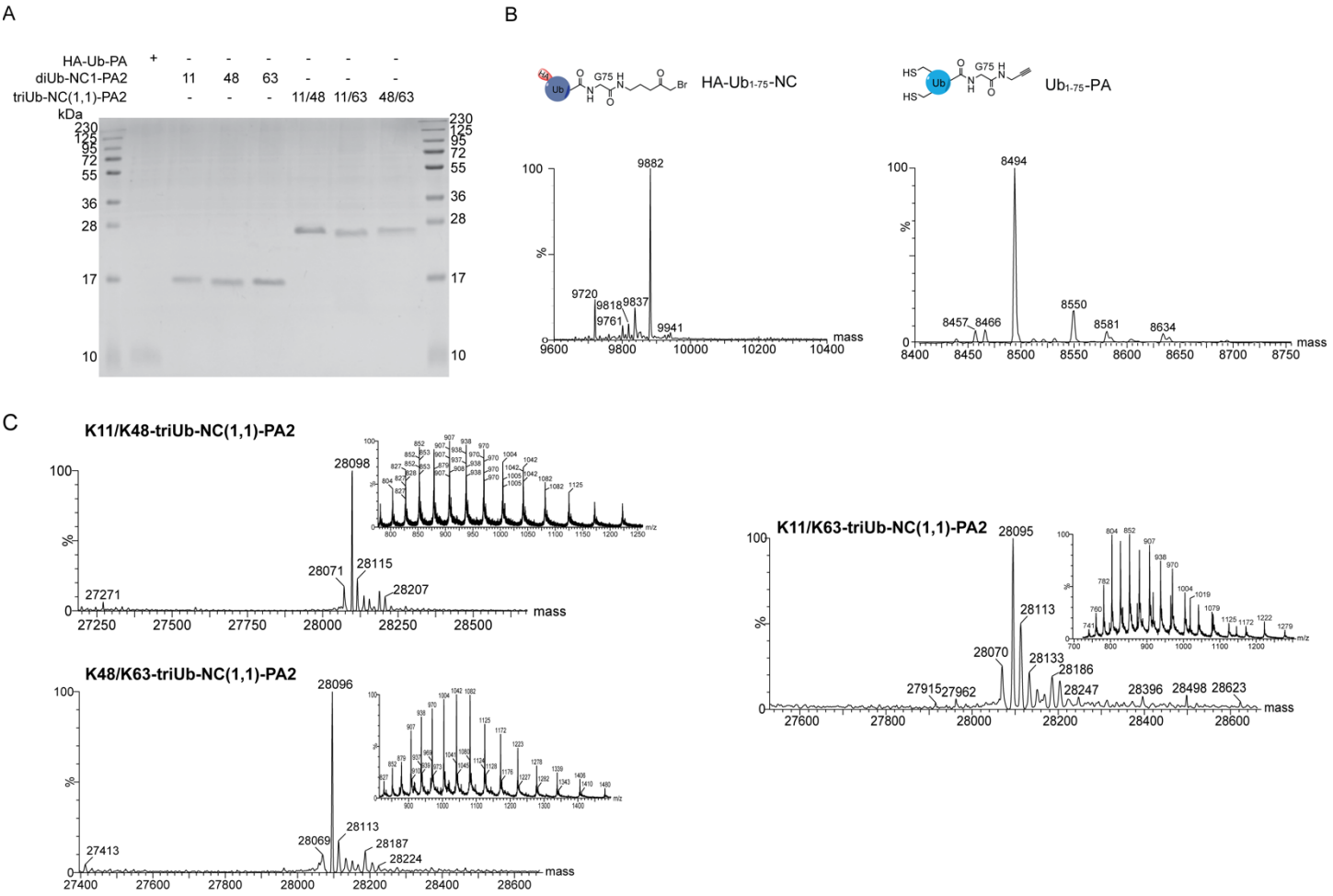

Figure S1. (A) SDS-PAGE gel analysis of diUb-PA and triUb-PA probes as indicated. (B) MS analysis of intermediates of branched triUb probe generation (calculated mass for HA-Ub_{1-75}-NC: 9882 Da; mutant Ub_{1-75}-PA: 8495 Da). (C) MS analysis of the three branched triUb probes (calculated mass: 28,097 Da).

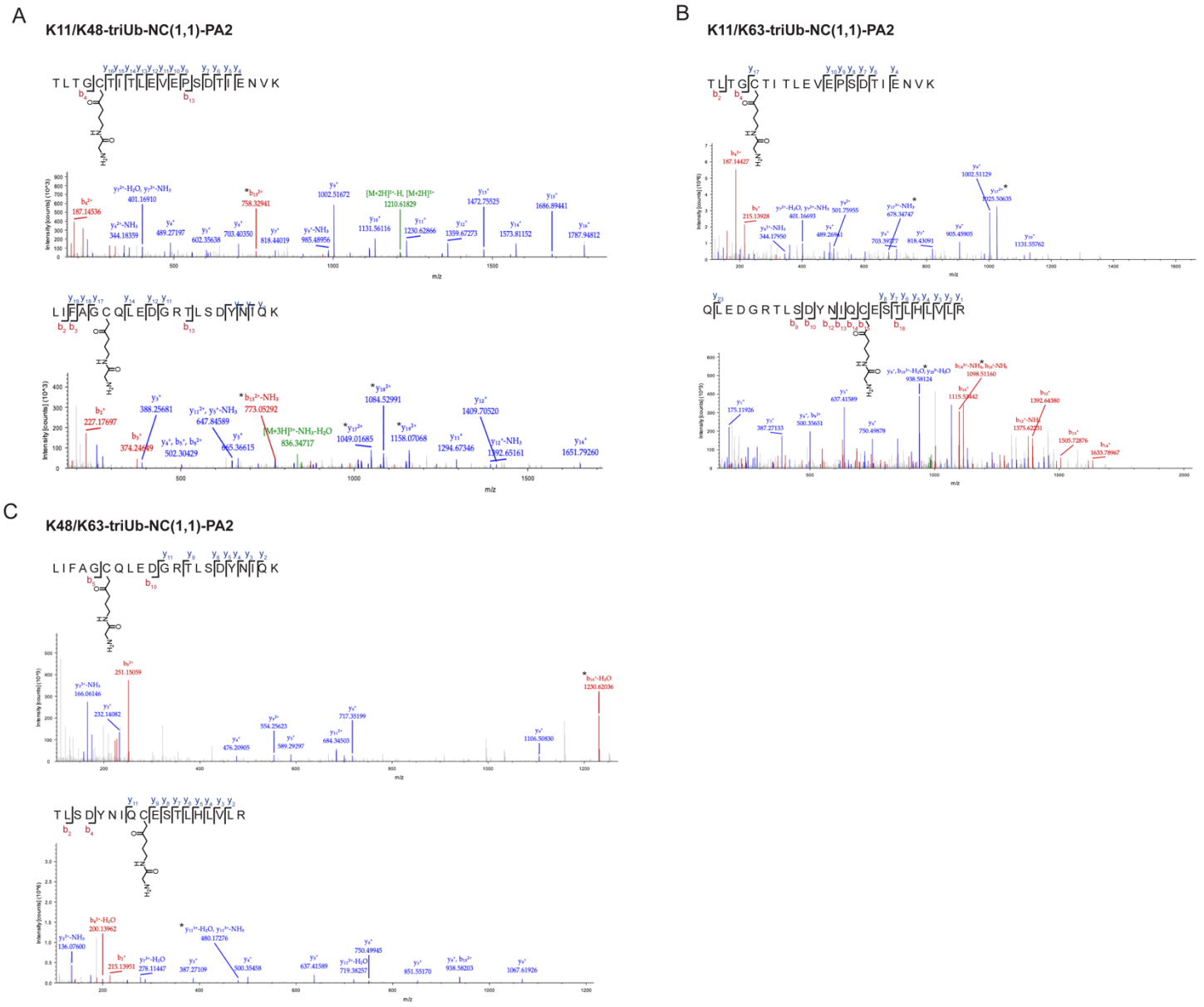

Figure S2. Mass spectrometry analysis (MS/MS) of branched chain triUb-PA. (A) K11/K48 triUb-NC(1,1)-PA2, (B) K11/K63 triUb-NC(1,1)-PA2, (C) K48/K63 triUb-NC(1,1)-PA2 . The selected peptides were obtained from MS/MS spectra of pulldown experiments using the corresponding branched triUb probes. Asterisk indicates fragments containing the corresponding branching sites.

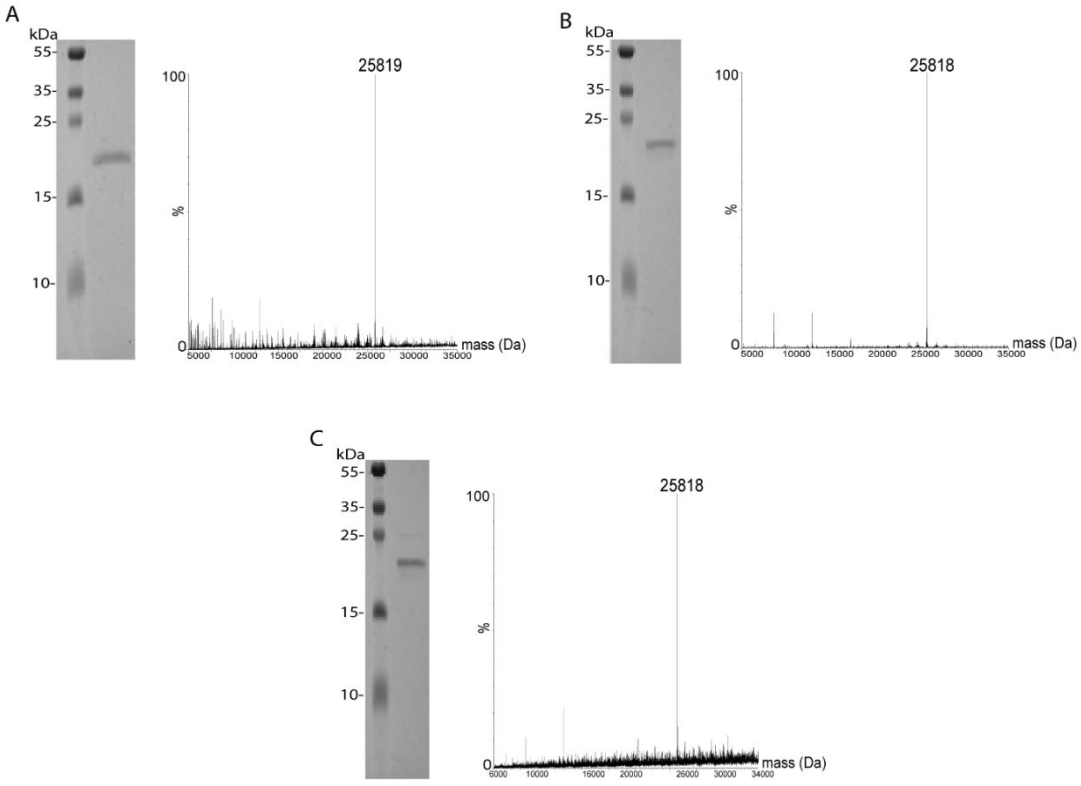

Figure S3. SDS-PAGE and mass spectrometry analysis (intact mass) of branched triUb-(1,0) species with (A) K11/K63, (B) K11/K48 and (C) K63/K48 linkages respectively. The calculated molecular weight of the triUb-(1,0) is 25,819 Da.

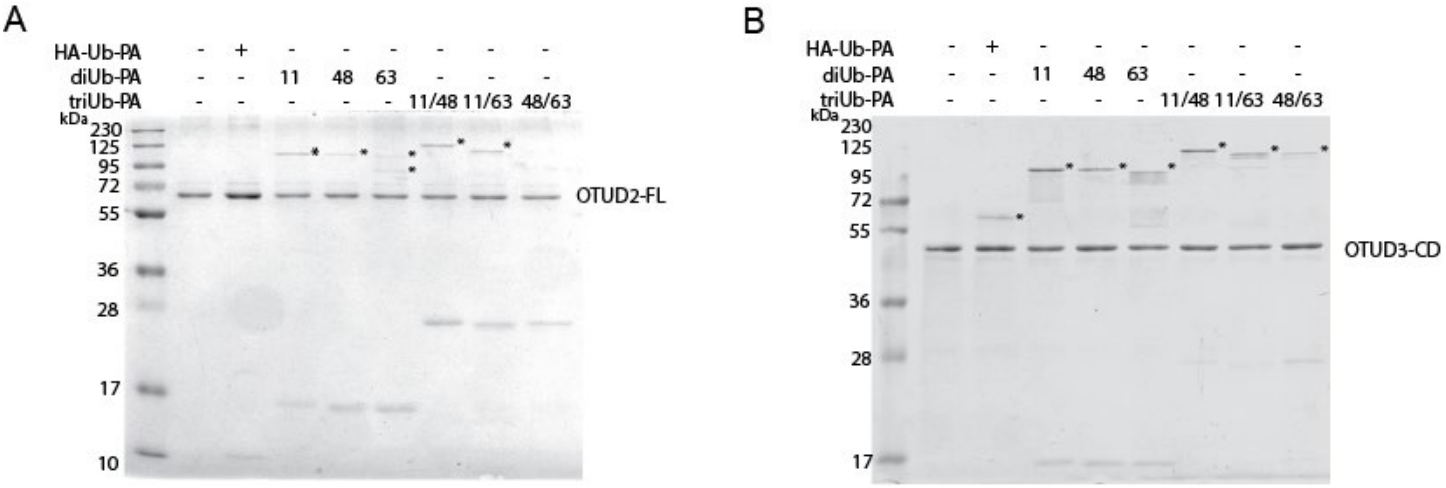

Figure S4. *In-vitro* labeling of purified DUBs (A) OTUD2-FL and (B) OTUD3-CD with mono-, di- and branched triUb-PA probes as indicated. SDS-PAGE gels were stained with Coomassie brilliant blue dye. Asterisks indicate detected labeling bands.

Gene	Beads	Beads	Beads	HA-Ub-PA	HA-Ub-PA	HA-Ub-PA	K11-diUb-PA	K11-diUb-PA	K11-diUb-PA	K48-diUb-PA	K48-diUb-PA	K48-diUb-PA	K63-diUb-PA	K63-diUb-PA	K63-diUb-PA	K11/K48-triUb-PA	K11/K48-triUb-PA	K11/K48-triUb-PA	K11/K63-triUb-PA	K11/K63-triUb-PA	K11/K63-diUb-PA	K48/K63-triUb-PA	K48/K63-triUb-PA	K48/K63-triUb-PA
WRIP1	NaN	NaN	NaN	28.9533	NaN	NaN	30.9614	30.8036	30.7875	30.8633	25.2336	24.4499	30.6035	25.4118	25.97	31.7	26.6	27.3	30.5	30.3	30.5	29.7342	30.0009	30.0113
UIMC1	NaN	NaN	NaN	21.9	18.3	19.3	27.6	27.5	28.6	24.7	26.9	26.8	27.6	29.6	29.8	27.5	28.8	29.4	30.9	31.1	31.0	30.7	30.5	30.5
BRE	NaN	NaN	NaN	25.3	NaN	NaN	25.7	26.0	27.4	26.1	26.0	26.0	27.5	27.8	27.7	27.7	26.9	27.5	28.1	27.6	28.0	29.0	28.8	28.7
SQSTM	NaN	NaN	NaN	NaN	NaN	NaN	26.1	24.6	26.3	NaN	NaN	NaN	NaN	NaN	NaN	26.6	27.1	27.3	26.9	26.1	26.0	27.2	27.5	27.4
ASCC2	NaN	NaN	NaN	20.5	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	27.8	23.5	23.3	NaN	NaN	NaN	23.0	NaN	22.3	23.2	NaN	21.7
AN13A	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	24.6	NaN	NaN	NaN	NaN	27.1	26.3	27.2	NaN	NaN	NaN
UBE3A	NaN	NaN	NaN	24.1	24.8	22.6	NaN	NaN	NaN	23.0	NaN	NaN	24.4	23.8	24.0	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
ITCH	NaN	NaN	NaN	26.2383	23.9	24.0	NaN	NaN	NaN	NaN	NaN	NaN	23.4	23.6	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
UBE2N	NaN	NaN	NaN	25.9	25.5	25.3	NaN	NaN	NaN	24.0	23.8	NaN	25.4	25.1	25.4	23.4	NaN	NaN	25.4	25.3	25.3	NaN	NaN	NaN
UB2E1	NaN	NaN	NaN	27.9	26.8	26.8	NaN	26.5	26.1	28.0	26.8	26.9	27.8	26.3	26.2	27.9	27.0	28.1	28.8	27.8	29.2	28.0	28.4	28.1
UB2E2	NaN	NaN	NaN	25.1798	24.0449	24.1451	NaN	NaN	23.979	25.1	23.5	23.9	24.8	23.6	23.5	24.4	24.2	24.9	25.9	25.4	26.0	24.9	25.8	24.8
HUWE1	NaN	NaN	NaN	32.3932	31.8511	31.7457	34.4031	34.3	34.3	34.5	34.6	34.5	33.9	33.8	33.9	33.7	33.6	34.1	34.0	33.8	34.0	33.6	33.6	33.6
UB2D3	NaN	NaN	NaN	28.6582	28.348	28.182	27.7644	28.7	28.1	28.1	27.9	27.8	28.1	28.4	28.0	27.9	28.0	28.6	29.5	29.2	29.5	28.6	28.2	28.6
ADRM1	NaN	NaN	NaN	29.2037	28.694	28.8866	28.7576	28.6636	28.413	29.9067	30.846	30.4029	30.0	30.1	30.2	28.6	28.7	28.0	29.6	29.7	29.5	29.3	29.5	29.6
PSMD4	NaN	NaN	NaN	30.7175	30.4701	30.7869	30.0775	30.1895	29.9998	31.8099	32.4	32.2006	31.8	32.1	32.3	29.9	29.8	30.4	30.9	31.7	31.3	30.4	30.8	30.5
RO52	28.3808	28.4788	27.837	27.2	28.4	28.3	28.2	28.1	28.0	27.0	27.8	27.5	26.9	27.6	27.8	27.3	28.4	28.7	23.9	26.0	25.7	28.1	28.1	28.0
ZUFSP	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	25.2	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
ZRANB1	NaN	NaN	NaN	24.9	25.2	24.3	NaN	22.7	NaN	27.1	27.7	27.6	26.2	26.0	26.2	25.8	25.3	25.1	23.0	NaN	24.3	24.7	24.4	25.2
YOD1	NaN	NaN	NaN	28.9	28.3	28.5	29.3	28.8	28.8	28.2	28.4	28.4	29.0	29.1	29.2	29.5	29.3	29.8	30.3	30.4	30.2	30.1	30.3	30.3
VCPIP1	NaN	NaN	NaN	29.1	27.3	27.4	NaN	NaN	23.5	27.8	26.7	26.4	28.6	26.9	26.7	25.4	26.5	27.0	30.9	31.0	31.1	25.3	24.6	22.6
USP9X	NaN	NaN	NaN	33.3	32.4	31.9	29.4	29.4	29.1	33.3	32.5	32.5	33.5	32.3	32.3	28.2	25.5	26.3	27.4	25.7	28.6	31.4	31.3	31.2
USP8	NaN	NaN	NaN	27.6	NaN	NaN	27.2	27.3	27.0	27.1	25.9	25.9	29.7	28.8	29.0	NaN	NaN	NaN	27.6	27.2	26.7	NaN	NaN	NaN
USP7	NaN	NaN	NaN	31.7	30.9	30.8	31.8	31.6	31.6	32.5	32.4	32.3	32.8	32.4	32.5	30.7	30.0	30.1	32.5	31.8	32.5	30.9	30.6	30.6
USP54	NaN	NaN	NaN	NaN	24.0	24.3	24.8	NaN	25.2	NaN	24.7	24.0	NaN	24.7	24.8	24.0	24.8	23.8	24.3	NaN	22.7	24.4	24.7	23.9
USP5	NaN	NaN	22.4	30.6	28.6	28.3	32.9	32.6	32.6	33.3	33.0	33.1	33.1	32.6	32.7	32.0	31.9	32.5	34.4	34.0	34.3	32.2	32.3	32.3
USP48	NaN	NaN	NaN	30.2	28.9	28.8	25.3	25.3	26.5	29.5	28.6	28.6	29.4	28.2	27.9	NaN	NaN	NaN	NaN	NaN	NaN	22.7	NaN	NaN
USP47	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	27.8	27.4	27.0	28.4	27.6	27.9	NaN	NaN	24.7	28.6	28.5	29.1	NaN	NaN	NaN
USP42	23.5	NaN	NaN	NaN	NaN	NaN	NaN	22.6	23.0	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	22.8	22.5
USP40	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	23.8	NaN	NaN	26.1	24.1	NaN	NaN	NaN	NaN	25.5	24.5	26.6	NaN	NaN	23.7
USP4	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	25.6	26.1	24.5	NaN	NaN	NaN	28.2	27.4	28.0	NaN	NaN	NaN
USP38	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	23.2	NaN	NaN	24.3	24.0	NaN	NaN	NaN	25.4	24.3	25.5	NaN	NaN	NaN
USP37	NaN	NaN	NaN	28.0	25.8	26.5	26.1	25.6	27.2	27.7	28.8	28.4	27.7	28.3	28.1	27.0	24.7	23.8	25.9	25.0	26.2	24.8	25.4	25.0
USP35	NaN	NaN	NaN	21.3	NaN	NaN	NaN	NaN	NaN	NaN	21.6	21.9	21.3	21.9	21.6	NaN	NaN	NaN	23.1	21.9	22.7	NaN	NaN	21.3
USP34	NaN	NaN	NaN	24.0	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
USP28	NaN	NaN	NaN	29.3	28.8	28.5	25.8	24.1	26.3	26.5	27.8	27.4	29.8	28.7	29.4	23.6	NaN	22.8	29.0	28.7	29.4	NaN	NaN	22.7
USP27X	NaN	NaN	NaN	23.7	23.8	23.5	NaN	NaN	NaN	NaN	22.6	22.5	NaN	23.3	24.1	NaN	NaN	NaN	NaN	NaN	22.4	NaN	NaN	NaN
USP25	NaN	NaN	NaN	29.8	28.1	28.5	22.0	22.4	22.4	NaN	NaN	NaN	30.5	30.6	30.2	NaN	NaN	NaN	23.6	25.7	28.6	NaN	NaN	NaN
USP24	NaN	NaN	NaN	26.8	23.9	NaN	23.4	23.1	24.2	31.6	30.7	31.0	31.3	29.8	29.2	NaN	NaN	NaN	23.2	NaN	24.9	27.0	24.7	25.6
USP22	NaN	NaN	NaN	26.9	24.7	24.5	25.1	25.2	25.0	23.7	NaN	NaN	26.2	24.8	24.8	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
USP21	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	24.2	NaN	NaN	NaN	NaN	NaN	24.1	NaN	NaN	NaN	NaN	NaN	NaN
USP2	NaN	NaN	NaN	35.0	23.7	NaN	NaN	NaN	NaN	30.5	24.9	24.3	27.6	NaN	NaN	33.0	NaN	24.4	NaN	NaN	NaN	NaN	NaN	NaN
USP19	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	24.5	NaN	25.2	NaN	NaN	NaN
USP16	NaN	NaN	NaN	28.3	26.6	26.0	31.9	32.1	31.9	30.8	31.1	31.0	30.6	31.0	31.2	30.9	30.6	30.9	30.4	30.4	30.3	31.1	31.1	31.1
USP15	NaN	NaN	NaN	28.0	25.7	23.7	27.1	27.1	26.3	29.3	29.1	28.9	31.8	31.3	31.3	22.5	NaN	NaN	31.2	30.8	31.3	22.2	NaN	NaN
USP14	NaN	NaN	NaN	31.6	30.9	31.0	31.1	31.1	30.9	32.4	33.0	32.8	32.4	32.6	32.6	30.5	30.3	30.9	31.8	31.7	31.8	31.3	31.5	31.5
USP10	NaN	NaN	NaN	29.1	26.2	26.4	26.2	NaN	26.0	NaN	NaN	NaN	30.3	28.7	28.4	NaN	NaN	NaN	27.8	27.1	27.0	NaN	NaN	NaN
USP1	NaN	NaN	NaN	NaN	NaN	NaN	23.1	NaN	NaN	26.7	23.6	24.5	26.8	23.3	24.1	25.1	23.2	NaN	NaN	23.2	24.2	NaN	NaN	NaN
UCHL5	NaN	NaN	NaN	32.0	31.4	31.1	31.6	31.6	31.6	32.8	33.0	33.0	32.5	32.3	32.4	31.5	31.2	31.8	31.6	31.6	31.9	32.3	32.6	32.2
UCHL3	NaN	NaN	NaN	26.8	27.4	27.6	29.6	26.2	25.3	26.8	27.5	26.7	27.1	26.8	27.1	27.9	27.8	28.0	31.3	31.3	31.1	27.1	27.5	27.6
UCHL1	25.0	NaN	NaN	32.7	32.3	32.6	30.4	30.4	30.2	31.4	31.2	31.6	32.2	32.2	32.2	30.7	30.6	30.7	31.0	31.2	30.7	31.1	30.8	30.7
OTUD7B	NaN	NaN	NaN	29.7	28.2	28.2	27.6	28.0	28.0	29.6	28.9	28.8	29.6	29.0	29.1	30.0	28.7	29.4	30.1	30.0	30.2	29.6	29.7	29.7
OTUD6B	NaN	NaN	NaN	31.3	30.6	31.0	29.4	29.1	29.2	30.3	30.1	30.0	30.1	30.1	30.4	29.2	28.6	29.1	28.9	28.9	28.8	30.6	30.4	30.2
OTUD5	NaN	NaN	NaN	28.0	27.4	26.3	NaN	24.9	NaN	29.2	29.7	29.6	27.8	27.5	28.1	28.6	28.9	28.8	29.3	29.2	28.9	26.9	26.6	27.6
OTUD4	NaN	NaN	NaN	28.5	28.7	29.1	22.2	23.1	22.6	28.3	28.2	28.3	28.2	29.0	29.0	26.5	27.3	26.9	30.0	30.0	30.1	27.6	26.2	27.1
OTUD3	NaN	NaN	NaN	28.0	28.8	28.8	28.9	28.6	28.4	27.5	28.5	28.3	26.9	27.9	28.2	27.7	28.8	28.9	27.7	26.7	27.8	28.6	28.7	28.5
OTUD1	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	24.6	25.3	25.0	NaN	NaN	NaN	NaN	24.7	NaN	NaN	NaN	NaN	NaN	NaN	NaN
OTUB2	NaN	NaN	NaN	24.0	NaN	NaN	NaN	NaN	NaN	23.9	NaN	NaN	23.9	NaN	NaN	24.4	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN
OTUB1	NaN	NaN	NaN	32.9	31.9	31.8	31.6	31.6	31.4	32.7	31.5	31.9	32.8	31.8	31.7	32.4	31.9	33.0	33.6	33.2	33.2	32.3	32.6	32.4
JOSD2	NaN	NaN	NaN	25.8	25.6	24.9	NaN	NaN	NaN	24.9	25.5	25.6	25.3	24.8	24.6	NaN	NaN	NaN	NaN	NaN	NaN	24.5	24.3	NaN
BRCC3	23.5	24.1	24.2	26.2	24.2	24.8	28.5	28.2	28.3	28.0	27.4	27.7	28.6	28.9	28.9	28.9	28.4	28.8	29.7	29.5	29.8	30.2	29.9	29.8

Log$_2$(LFQ)
34
28
22

Figure S5. Pre-imputation LFQ values color coded according to Log$_2$(LFQ) of major UBD-containing protein interactors and DUBs present in any of the eight parallel pulldowns.

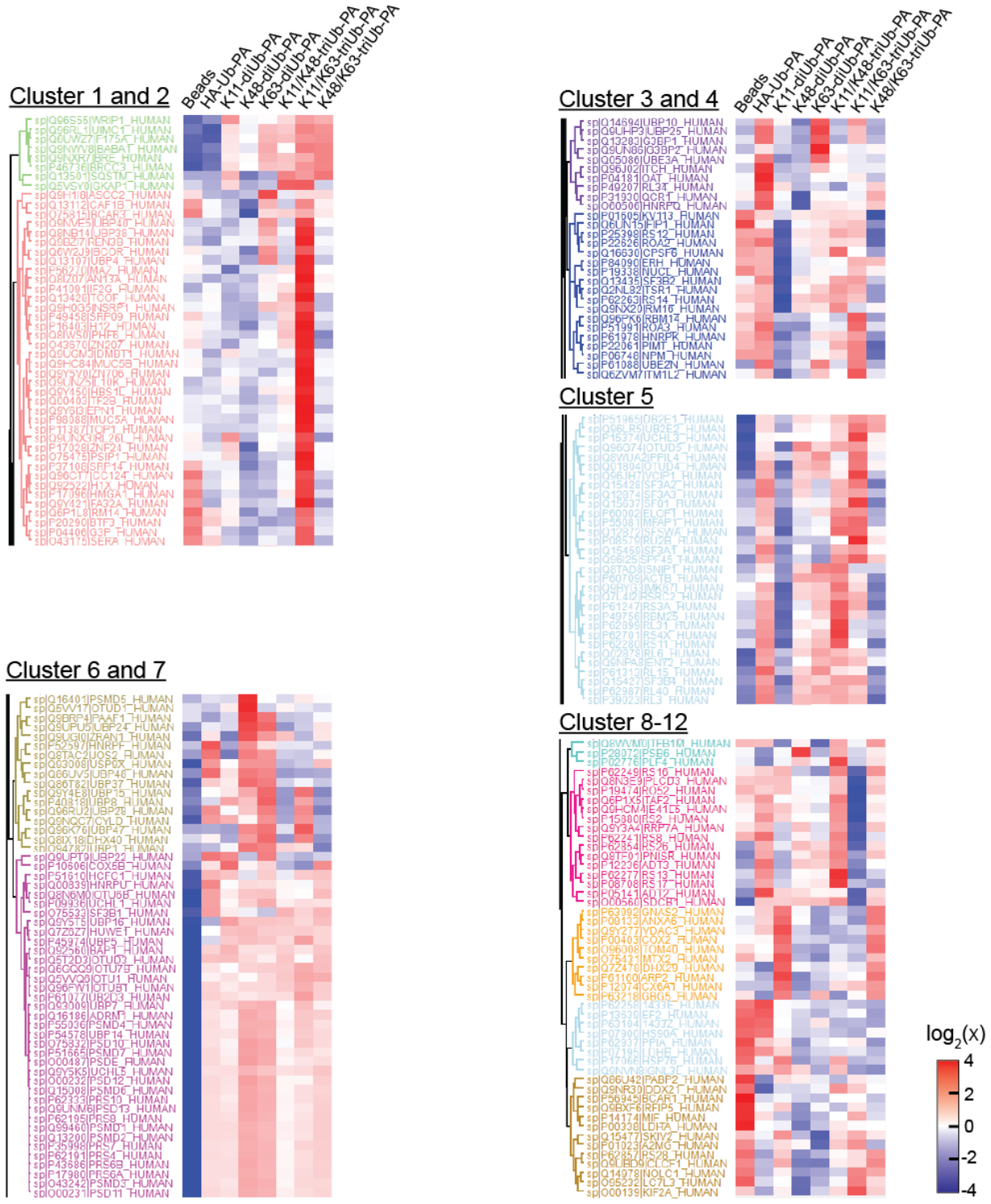

Figure S6. Hierarchical clustering of enriched proteins using branched triUb-PA probes in comparison to the monoUb-PA and diUb-PA probes. The protein names are given in each of the clusters. The heatmap reflects a Z-score normalization of $\log_2$(LFQ) value of a captured protein by the ubiquitin probes used in the parallel pulldown.

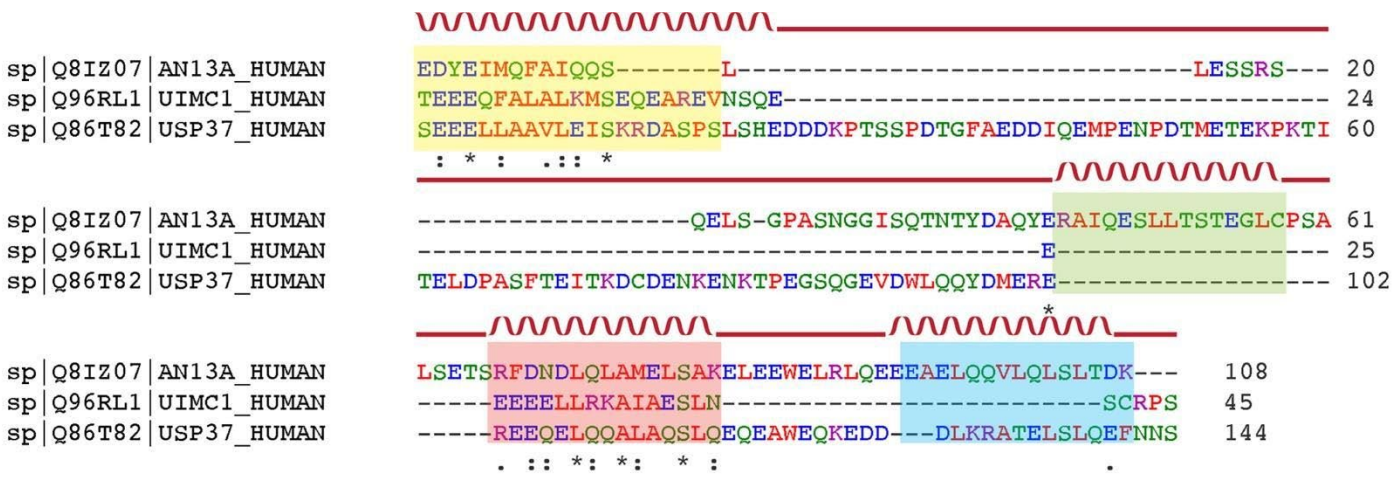

Figure S7. Clustal alignment of the identified UIM-containing sequences in UIMC1/Rap80, AN13A and USP37. Colored shading represents predicted UIM sequences. Secondary structure was predicted using AlphaFold modeling[7,8].

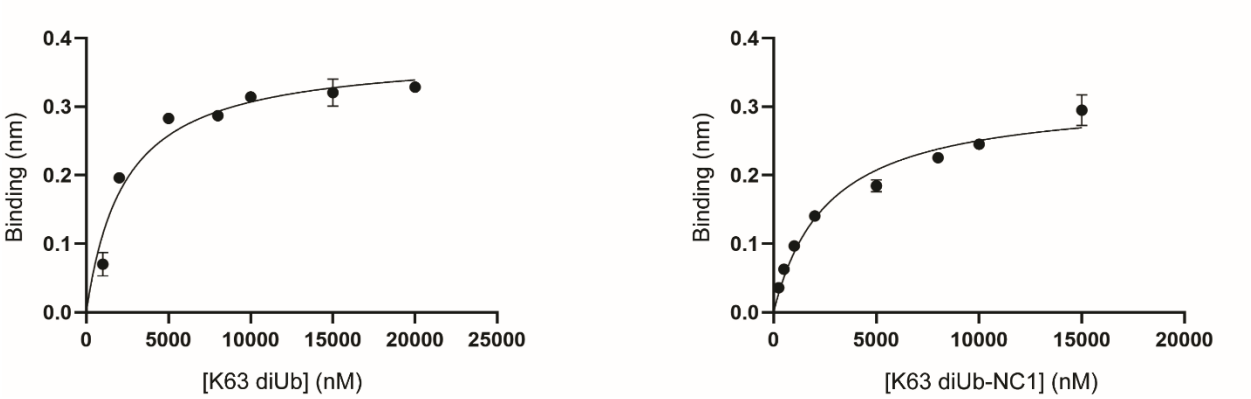

Figure S8. Comparison of K63-diUb with a native linkage (K63 diUb) to the K63-diUb containing the thioether linkage (K63 diUb-NC1) in binding to UIMC1$_{76\text{-}128}$. The K_d of UIMC1$_{76\text{-}128}$ binding to the Ub species was determined by fitting the maximum BLI binding observed versus the concentration of Ub species used in the BLI binding experiment. UIMC1$_{76\text{-}128}$ binds K63-diUb with a K_d of 2.34 ± 0.42 μM, in comparison to a K_d of 2.63 ± 0.41 μM for K63-diUb-NC1.

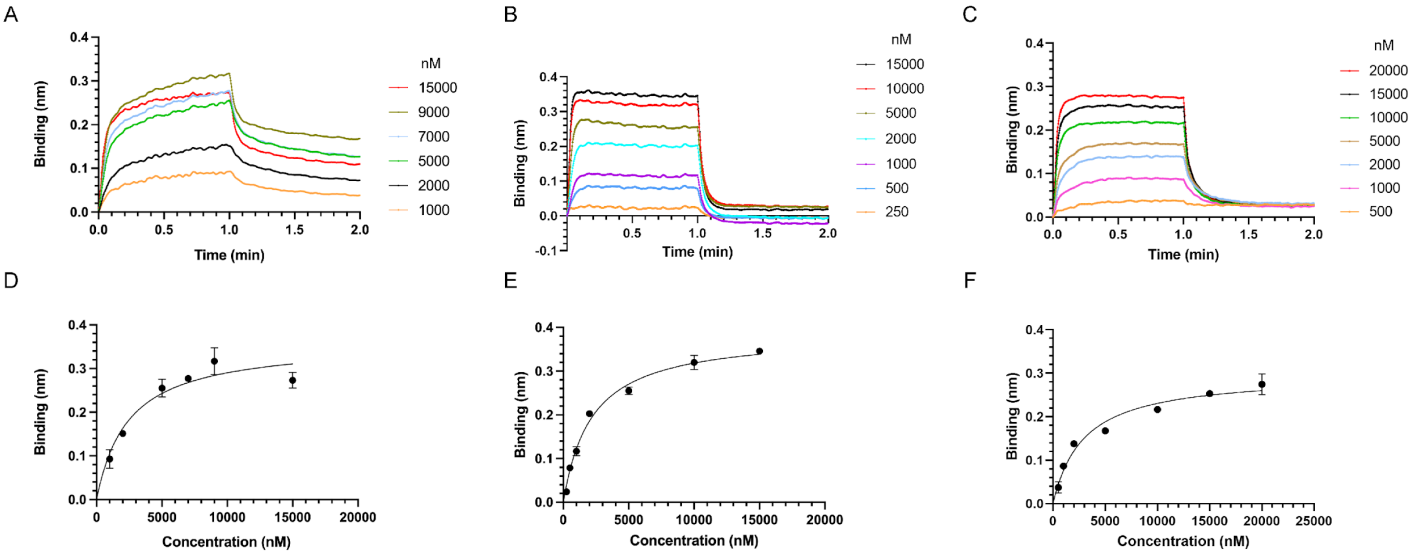

Figure S9. BLI traces of diUb and branched triUb(1,0) of different linkages binding to UIMC1$_{76\text{-}128}$ immobilized onto the BLI sensor tip surface. BLI traces averaged from two repeats are shown for concentration series of (**A**) K48/K63-triUb-(1,0); (**B**) K63-diUb; (**C**) K11-diUb. The K_d of binding was determined by fitting the maximum BLI binding observed versus the concentration of Ub species used in the BLI binding experiment for (**D**) K48/K63-triUb-(1,0), (**E**) K63-diUb and (**F**) K11-diUb.

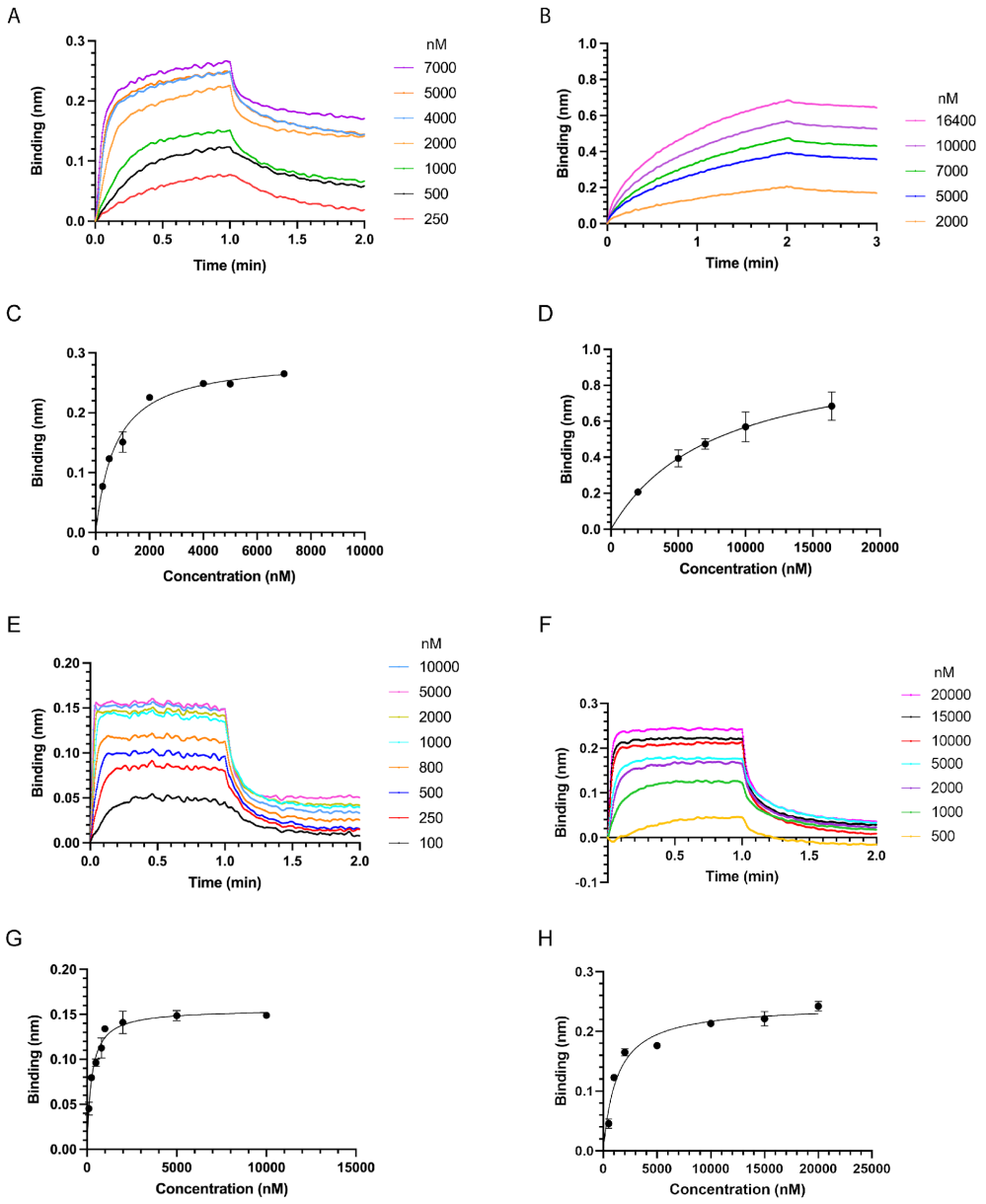

Figure S10. AN13A$_{479-594}$ binding to diUb and triUb(1,0) of indicated linkages. BLI traces averaged from two repeats are shown for concentration series of (**A**) K48/K63-triUb-(1,0); (**B**) K48/K11-triUb-(1,0); (**E**) K63-diUb; (**F**) K11-diUb. The K_d of AN13A$_{479-594}$ binding to the different Ub species was determined by fitting the maximum BLI binding observed versus the concentration of Ub species used in the BLI binding experiment for (**C**) K48/K63-triUb-(1,0), (**D**) K48/K11-triUb-(1,0); (**G**) K63-diUb and (**H**) K11-diUb.

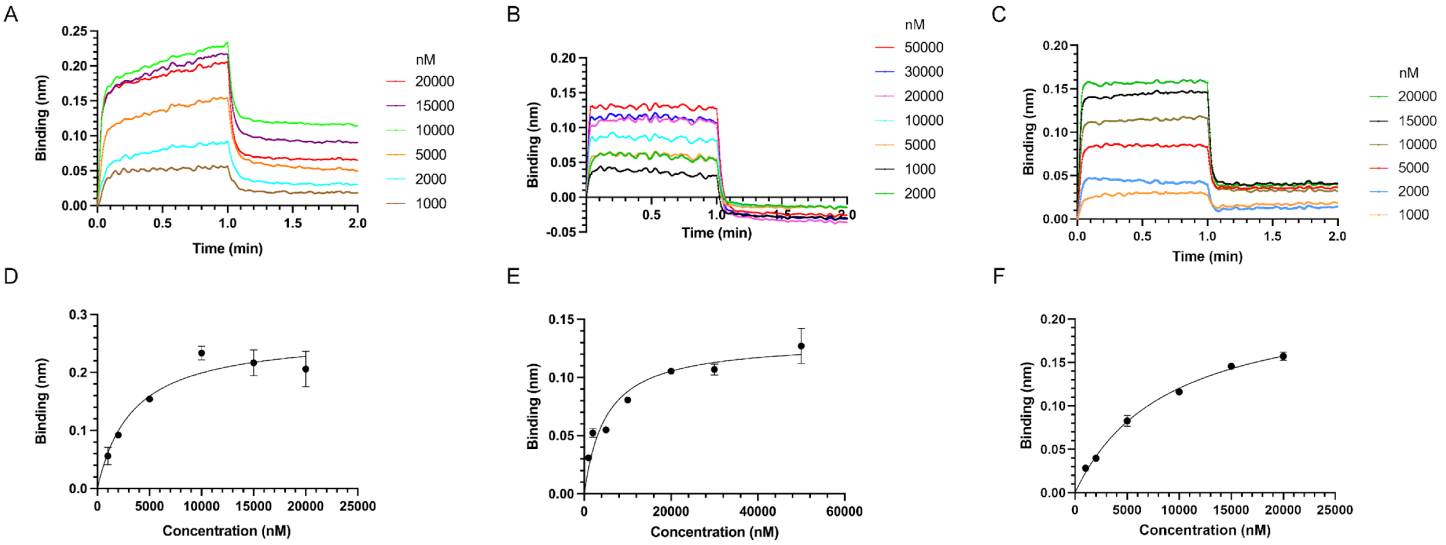

Figure S11. USP37$_{700-851}$ binding to diUb and triUb(1,0) of indicated linkages. BLI traces averaged from two repeats are shown for concentration series of (**A**) K63/K11-triUb-(1,0); (**B**) K63-diUb; (**C**) K11-diUb. The K_d of binding was determined by fitting the maximum BLI binding observed versus the concentration of Ub species used in the BLI binding experiment for (**D**) K63/K11-triUb-(1,0), (**E**) K63-diUb and (**F**) K11-diUb.

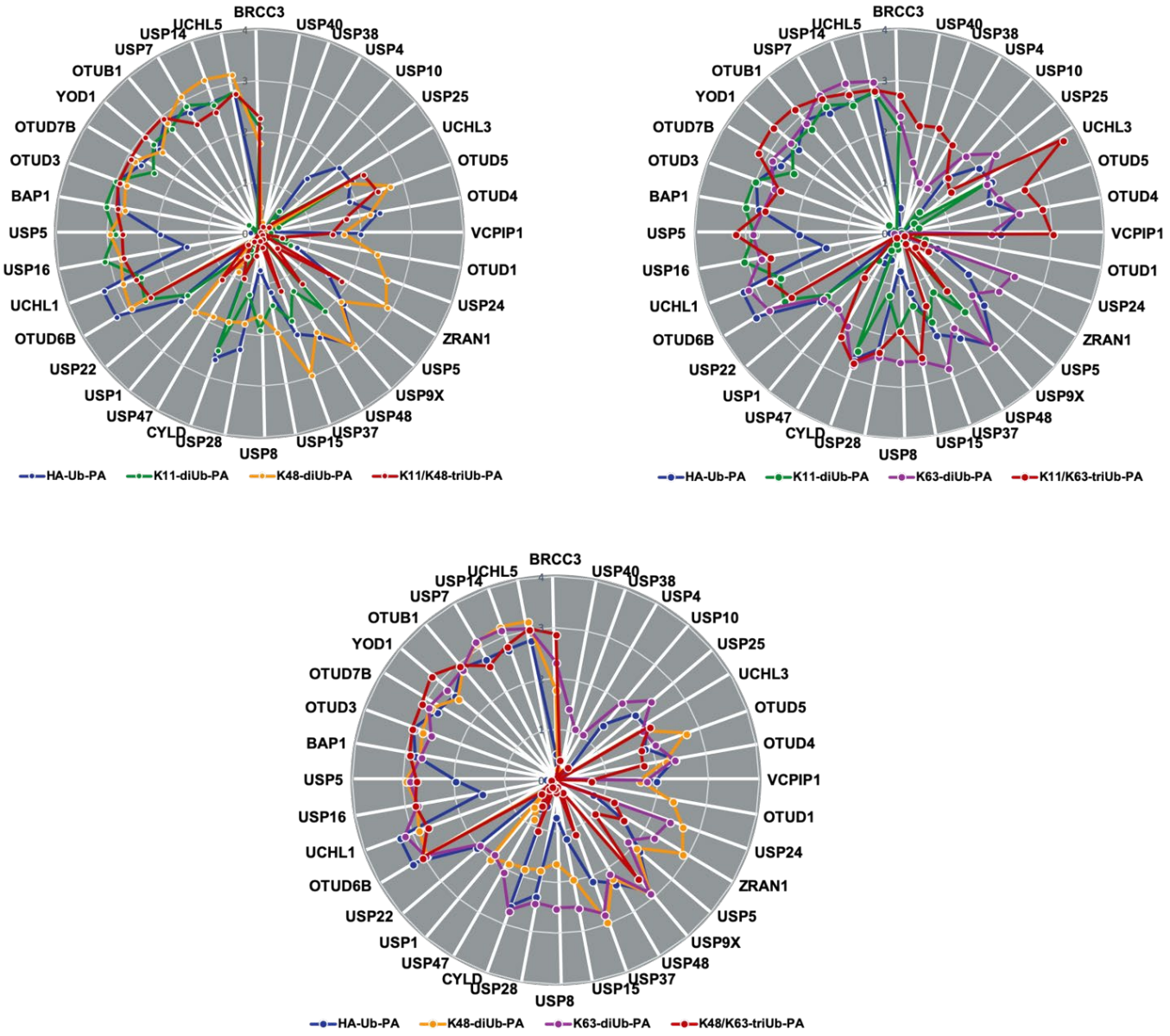

Figure S12. Spiderplot analysis of DUBs enriched by monoUb-PA, di-Ub-PA and branched triUb-PA probes of different linkages.

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