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Supplementary information

A bifunctional molecule-assisted synthesis of mimics for use in probing the ubiquitination system

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Supporting Information

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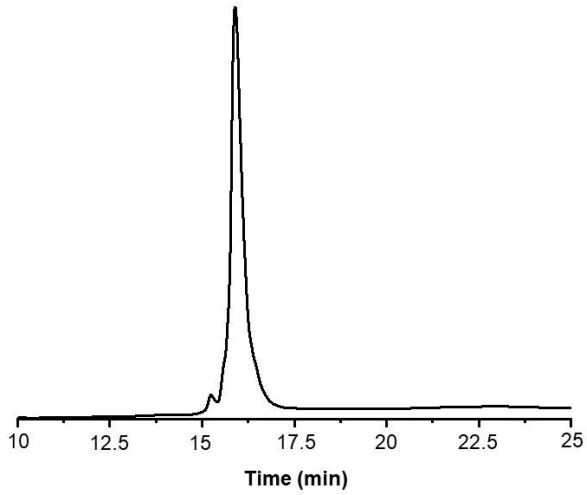
A)



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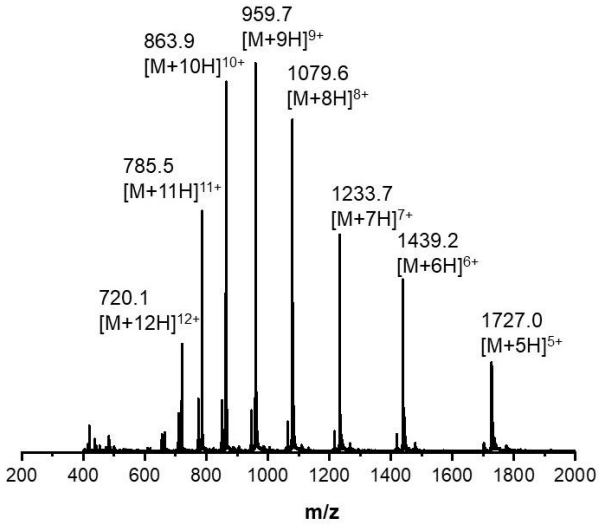
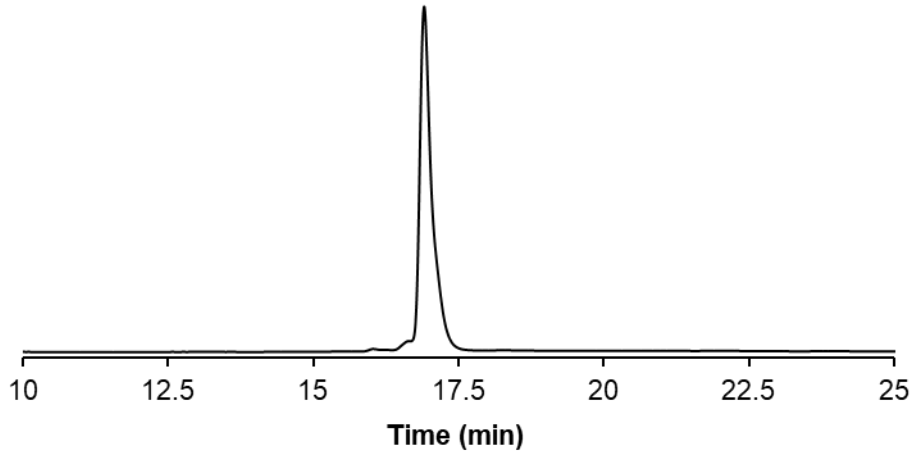


Supplementary Figure 1 | Synthesis of Ub75-MesNa. **A**) RP-HPLC trace (214nm) and **B**) ESI-MS of purified Ub75-MesNa. Calculated Mass:8630.1, Deconvoluted observed Mass: 8629.2

A)



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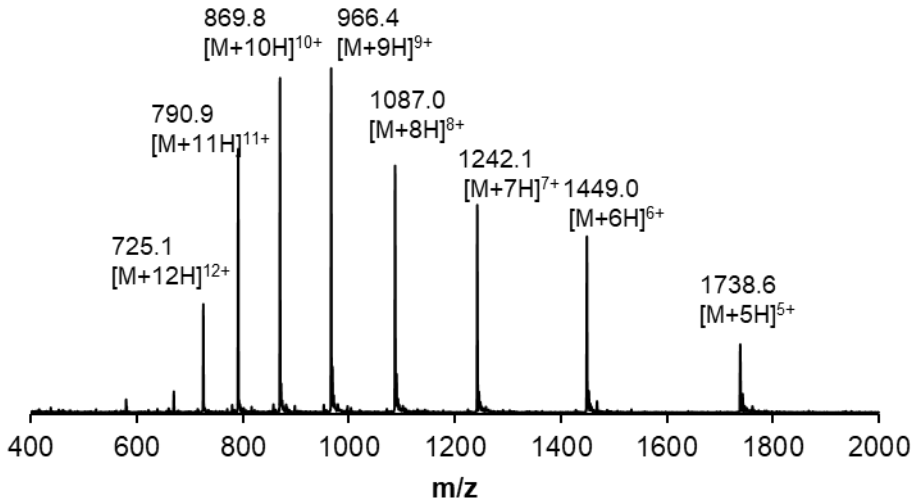
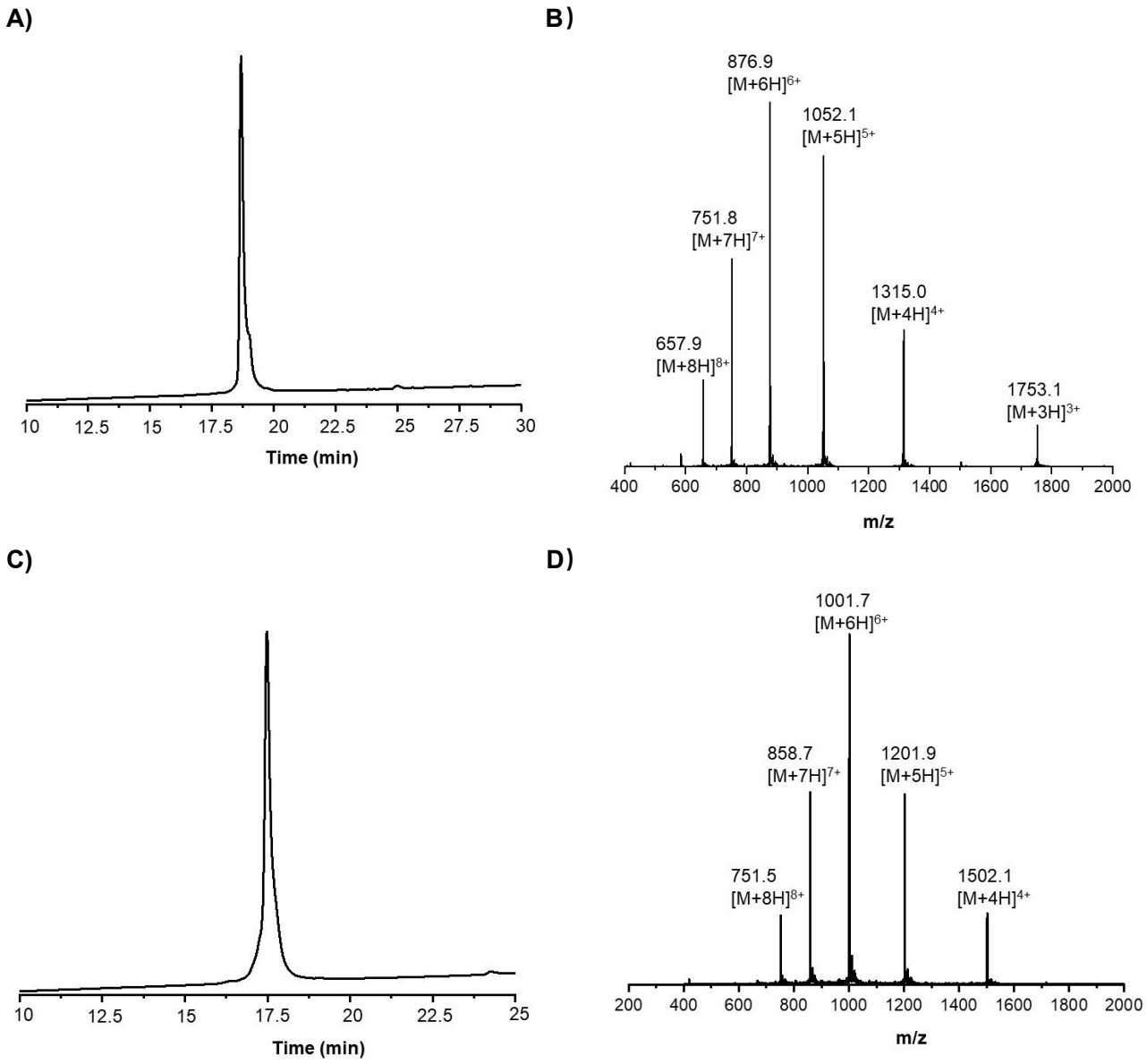
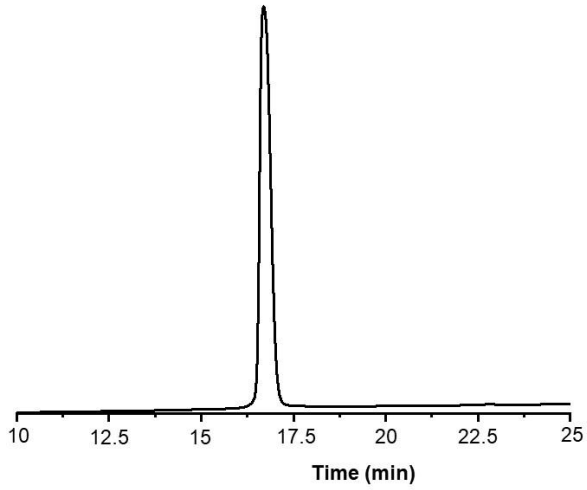


Supplementary Figure 2| Synthesis of Ub76-MesNa. **A**) RP-HPLC trace (214 nm) and ESI-MS of purified Ub76-MesNa. **B**) RP-HPLC trace (214 nm) and ESI-MS of purified Ub76-MesNa. Calculated Mass: 8688.1, Deconvoluted observed Mass: 8688.0



Supplementary Figure 3| Synthesis of peptide fragments by Fmoc-SPPS. **A**) RP-HPLC trace (214nm) and **B**) ESI-MS of purified Ub 1-47 $NHNH_2$. Calculated Mass:5256.1, Deconvoluted observed Mass: 5255.9. C) RP-HPLC trace (214nm) and **D**) ESI-MS of purified Y/degron-Ub[48C-76]. Calculated Mass:6004.8, Deconvoluted observed Mass: 6004.2

A)



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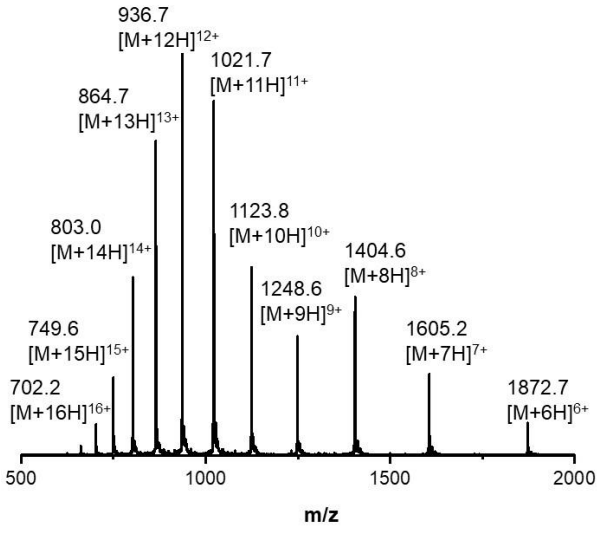
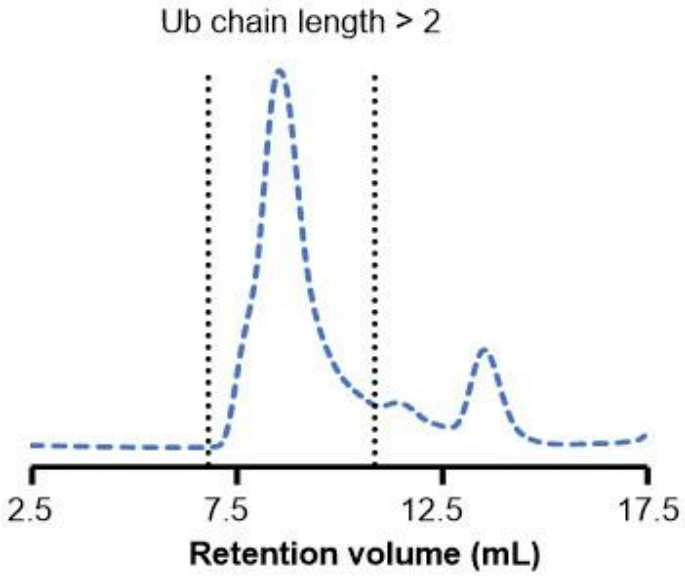


Supplementary Figure 4| Synthesis of Y/degron-Ub(K48C). **A**) RP-HPLC trace (214nm) and **B**) ESI-MS of purified Y/degron-Ub(48C). Calculated Mass: 11228.1, Deconvoluted observed Mass: 11228.8



Supplementary Figure 5| Refolded biotinylated K29-Ub chain mimic by SEC on a Superdex 75 10/300 GL column. Pool Fractions representing the more than 2 Ub moieties were collected for affinity enrichment by streptavidin beads.

Supplementary Method 1| Identification of the crosslinking site between Otud2 and K27-CAET diUb

Gel bands of the crosslinked Otud2-Ub-^{K27}Ub were excised and de-stained. Proteins were digested overnight at 37 °C with pepsin in 0.1% FA overnight. Peptides were extracted from the gel in 50% acetonitrile/1% trifluoroacetic acid (TFA) for 30 min and centrifuged in a SpeedVac to reduce the volume prior to MS analysis. Note that no reductant was used in these processes in order to determine the disulfide crosslinking site between Otud2 and K27-CAET diUb.

For LC-MS/MS analysis, tryptic peptides were separated by a gradient of acetonitrile in 0.1% (v/v) FA at a flow rate of 0.300 μL/min with a Thermo-Dionex Ultimate 3000 HPLC system, which was directly interfaced with the Thermo Orbitrap Fusion MS. The analytical column was a homemade fused silica capillary column (75 μm ID, 150 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 Å, 5 μm; Varian, Lexington, MA). The Orbitrap Fusion MS was operated in the data-dependent acquisition mode using Xcalibur3.0 software, and there is a single full-scan mass spectrum in the Orbitrap (350-1550 m/z, 120,000 resolution) followed by 3 seconds of data-dependent MS/MS scans in an Ion Routing Multipole at 30% normalized collision energy (HCD).

As for data analysis, the MS/MS spectra from each LC-MS/MS run were searched using pLink2 software. The linker information was set as a mass of 371.174 ($C_{14}H_{25}N_7O_3S$) between 2 Cys sites. pLink search parameters were as below: precursor mass tolerance of 20 ppm, fragment mass tolerance of 0.02 Da, peptide length minimum 6 amino acids and maximum 60 amino acids per chain, peptide mass minimum 600 and maximum 6,000 Da per chain, variable modification Oxidation[M] 15.994915, enzyme non-specific. The Otud2 and Ub protein sequences were downloaded from Uniprot.

Supplementary Method 2| Hydrogen–deuterium exchange mass spectrometry (HDX-MS)

Deuterium-exchange reactions of Otud2 alone and decoupling intermediate Otud2-Ub-^{K27}Ub were initiated by diluting the protein to a final concentration of 18 μM with labeling buffer D_2O (20 mM HEPES, 150 mM NaCl, 99% D2O, pH 7.1). For all experiments, deuterium labelling was carried out at 25 °C at five time points: 0 s, 30 s, 90 s, 300 s and 24 h, in technical triplicate. The labelling reaction was quenched by the addition of an equal volume of ice-cold quench buffer (200 mM citric acid, 500 mM TCEP and 4 M Gn·HCl in 100% ddH_2O at pH 1.8).

The quenched protein samples were rapidly put on ice (each of 50 μL volume) and subjected to proteolytic cleavage with pepsin followed by RP-HPLC separation. In brief, 5 μL 1 μM pepsin solution was added for 2-min digestion. Then peptic peptides were placed into a Thermo-Dionex Ultimate 3000 HPLC system autosampler for injection.

Trapped peptides were subsequently eluted for 20 min using a gradient of acetonitrile in 0.1% (v/v) formic acid at a flow rate of 115 μl/min. Peptides were separated on a reverse-phase column (Acquity UPLC BEH C18 column, 1.7 μm, 2.1*50 mm, Waters, UK) and detected on a Q Exactive MS (Thermo) over an m/z of 350 to 2,000 (70,000 resolution) in the data-dependent acquisition mode using Xcalibur 2.0.0.0 software. MS was operated at a source temperature of 250 °C and a spray voltage of 3.0 kV.

Mass analysis of the peptide centroids was performed using Proteome Discoverer (Version PD1.4, Thermo-Fisher Scientific, USA). Set search criteria as follows: No enzyme; Max missed cleavages of 2; Precursor ion mass tolerances of 20 parts per million (ppm); 0.02 Da; Fragment ion mass tolerance of 0.02 Da. Only peptides with a peptide false discovery rate (FDR) < 1% were considered. The deuterium exchange levels were determined by subtracting the centroid mass of undeuterated peptide from the centroid mass of deuterated peptide using HDExaminer (Version PD1.4, Thermo-Fisher Scientific, USA). Heatmap was displayed to show, for each partially-deuterated experiment, the computed deuteration percentage at each residue, which was colored from blue (more protected from exchange compared to the previous state) to red (more accessible to solvent exchange).