Electronic Supplementary Material (ESI) for ChemComm.

Assembly of Branched Ubiquitin Oligomers by Click Chemistry

Xiaohui Zhao, Martin Scheffner and Andreas Marx

Departments of Chemistry and Biology, Konstanz Research School Chemical Biology, University of Konstanz, 78457 Konstanz, Germany

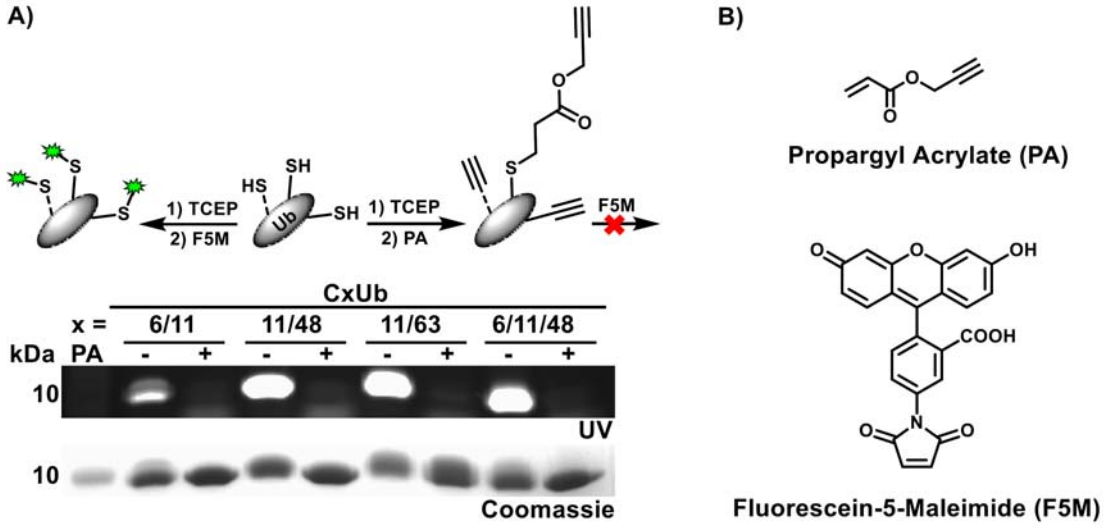


Fig. S1 Functionalization of CxUb (x = 6/11, 11/48, 11/63, 6/11/48) with alkynes. A) F5M labeling of PA-treated (+) and untreated (-) CxUb. The gel was visualized by UV light (302 nm; upper panel) followed by Coomassie blue staining (lower panel). B) Structure of chemicals used in the reaction.

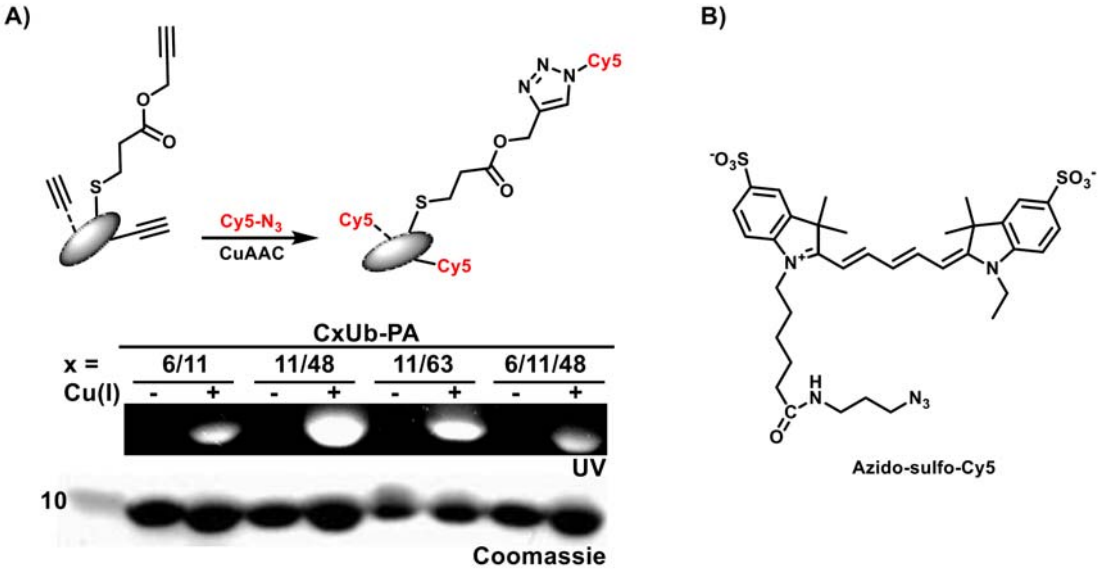


Fig. S2 Fluorescent labeling of CxUb-PA with Cy5. A) Click reaction with (+) and without (-) copper (I) catalyst. The gel was visualized under UV light followed by Coomassie blue staining. B) Chemical structure of azido-sulfo-Cy5.

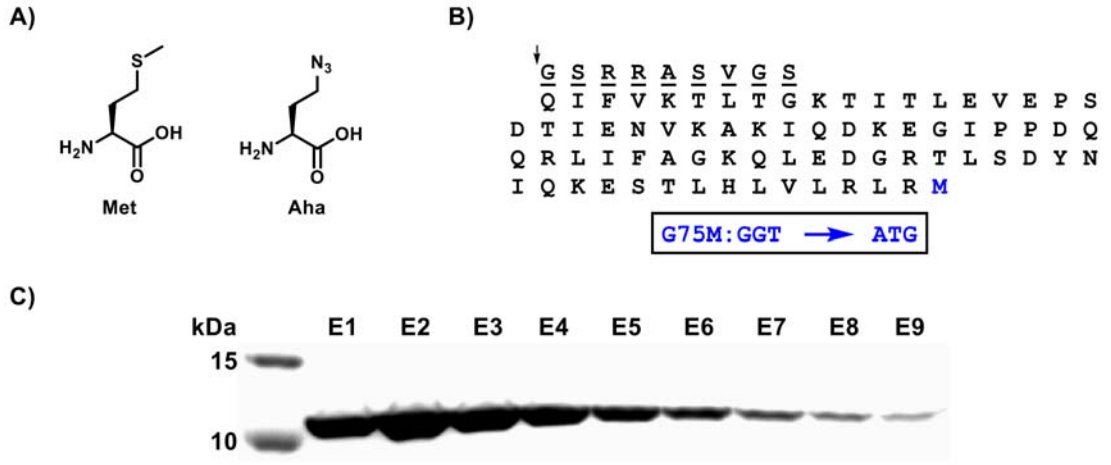
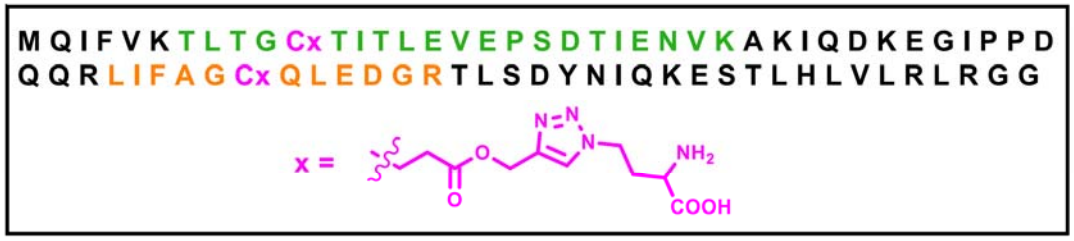
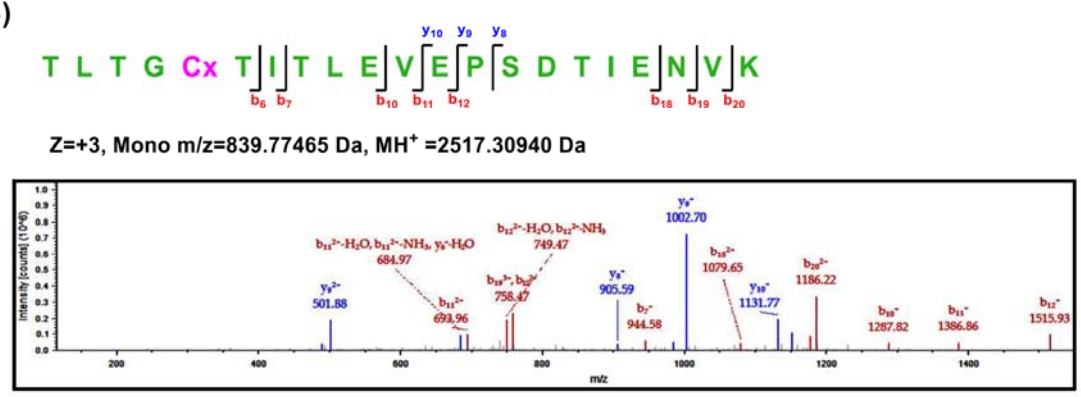


Fig. S3 Generation of azide-functionalized Ub. A) Chemical structures of methionine (Met) and azidohomoalanine (Aha). B) Sequence of Aha75Ub. Arrow indicates the thrombin cleavage site. The N-terminal extension is indicated in underlined letters. C) SDS-PAGE analysis of Aha75Ub present in different elution fractions. Aha75Ub fused to glutathione S-transferase (GST-TCS-Aha75Ub) was bound to glutathione agarose beads and eluted via thrombin-cleavage. Proteins were visualized by Commassie blue staining.

A)



B)



C)

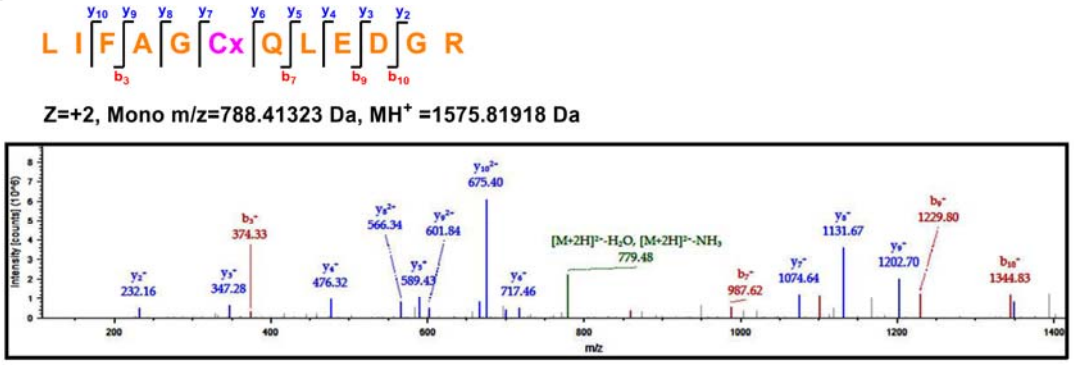


Fig. S4 Mass spectrometric analysis of Ub_2-$^{11/48}$Ub. A) Sequence of Ub with modifications (purple) at branching positions 11 and 48. Peptides from trypsin digestion are indicated in green and orange respectively. B) MS/MS analysis of the peptide containing a modification at position 11. C) MS/MS analysis of the peptide containing a modification at position 48.

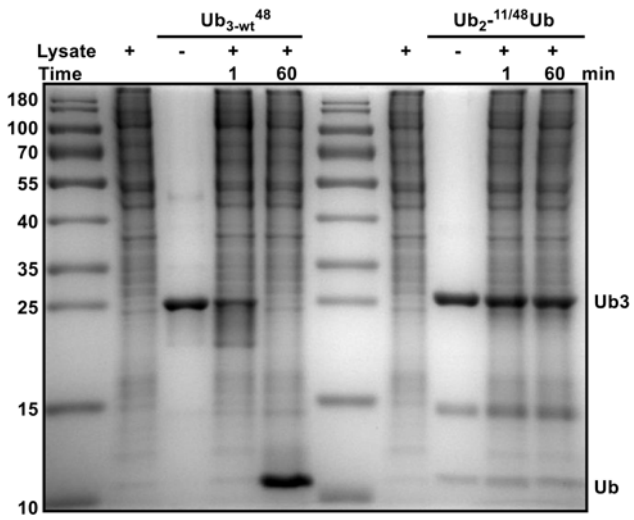


Fig. S5 SDS-PAGE analysis of the wild-type Ub trimer ($Ub_{3\text{-}wt}^{48}$) and the synthesized branched Ub trimer (Ub_2-$^{11/48}$Ub) incubated in the presence (+) and absence (-) of HEK293T cell lysate. Proteins were visualized by Commassie blue staining.

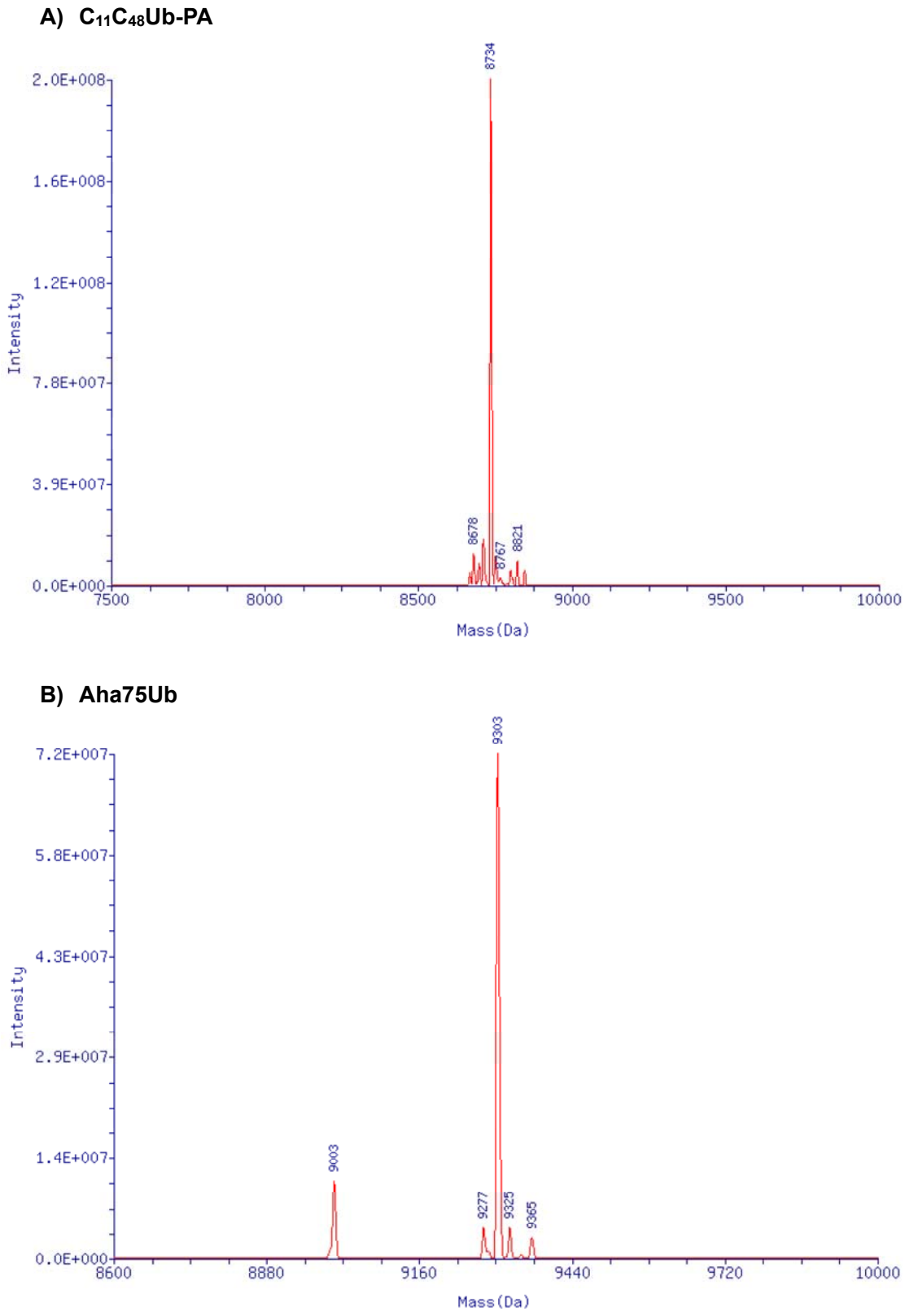


Fig. S6 Mass spectrometry analysis of A) $C_{11}C_{48}$Ub-PA and B) Aha75Ub.

Expression and purification of CxUb (x= 6/11, 11/48, 11/63, 6/11/48)

The cDNA encoding human CxUb (x= 6/11, 11/48, 11/63, 6/11/48) was cloned into the pET3a vector by *Nde* I / *BamH* I double digest and T4 DNA ligase ligation. *E.coli* BL21 (DE3) transformed with pET3a-CxUb was cultured in LB medium containing 100 mg/L carbenicillin at 37 ˚C overnight. Then, the pre-culture was transferred to pre-warmed fresh LB/carbenicillin to an initial OD_{600} value of 0.1. When the OD_{600} value reached 0.6, expression of CxUb was induced by addition of 1 mM IPTG and the culture was incubated for another 5 h at 37 ˚C.

Cells were pelleted, resuspended in 20 mM sodium acetate (pH 4.5), and lysed by sonication. The lysate was clarified by high speed centrifugation for 30 min. The supernatant was transferred to a new tube, heated at 65 ˚C for 20 min followed by high speed centrifugation. The supernatant was withdrawn, cooled down to 4 ˚C, and adjusted to a pH value of 4.5. Eventually, proteins were purified by cation exchange chromatography (HiTrap SP HP, 1 mL, GE) on an ÄKTA FPLC system. Gradient elution from 0% to 100% buffer B of a total volume of 90 mL was performed using 20 mM sodium acetate (pH 4.5) as buffer A and 20 mM sodium acetate (pH 4.5) supplemented with 1 M sodium chloride as buffer B. The isolated CxUb was dialysed against 20 mM Tris-HCl (pH 7.5). Protein concentration was measured by BCA assay (ThermoFischer).

Modification of CxUb by propargyl acrylate (PA)

100 µM CxUb were treated with 10 eq. TCEP in 20 mM Tris-HCl (pH 7.5) at 37 ˚C for 0.5 h. Then, CxUb/TCEP was diluted to 20 µM and incubated with 100 eq. propargyl acrylate (PA) in 20 mM Tris-HCl (pH 7.5)/MeCN = 9:1 at 25 ˚C by shaking at 180 rpm for 2 h. 20 µL reaction mixtures were withdrawn and reacted with 25 eq. fluorescein-5-maleimide (F5M) in the dark at 37 ˚C for 20 min. As control, the same amount of PA-untreated CxUb was incubated with F5M under the same conditions. All the samples were analyzed by SDS-PAGE and visualized under UV-light. The same gel was subsequently stained by Coomassie blue. From 1 L of bacterial culture, more than 10 mg of PA-modified Ub were obtained.

To remove excess PA, reaction mixtures were transferred to dialysis tubes (3500 MWCO) and dialyzed sequentially against 20 mM Tris-HCl (pH 7.0)/MeOH = 1:1, 20 mM Tris-HCl (pH 7.0)/MeOH = 4:1, and 20 mM Tris-HCl (pH 7.0). The precipitated proteins were redissolved in 20 mM Tris-HCl (pH 7.0) containing 6 M guanidine hydrochloride, refolded through dialysis against progressively decreasing concentrations of guanidine hydrochloride (3 M and 1.5 M), and finally in 20 mM Tris-HCl (pH 7.0). The PA-modified proteins (CxUb-PA) were lyophilized in aliquots and stored at 4 ˚C. Before use, the required amount of powder was dissolved in water and quantified by BCA protein assay.

Labeling of CxUb-PA with azido-sulfo-Cy5

20 µM CxUb-PA and 25 eq. azido-sulfo-Cy5 were mixed in 20 mM Tris-HCl (pH 7.0). 0.5 mM SDS and 5 mM THPTA (final conc.) were added, followed by argon flushing. Click reaction was initiated by 2.5 mM $Cu(MeCN)_4BF_4$, and samples were incubated on ice for 1 h. The control reactions were performed under the same conditions, but without Cu(I) catalyst. All the samples were analyzed by SDS-PAGE and visualized under UV-light. The

same gel was subsequently stained by Coomassie blue.

Expression and purification of Aha75Ub

The cDNA encoding human G75MUb (M1 and G76 deleted) was cloned into the pGEX2TK vector by *BamH* I / *EcoR* I double digest and T4 DNA ligase ligation. Methionine auxotrophic *E.coli* B834 (DE3) transformed with pGEX2TK-G75MUb was cultured in LB medium containing 100 mg/L carbenicillin at 37 ˚C overnight. Then, the pre-culture was diluted with NMM medium containing 0.06 mM methionine and 100 mg/L carbenicillin to an OD_{600} value of 0.1. Cells were grown at 37 ˚C until they reached an OD_{600} of approximately 1.3, harvested and resuspended in the same volume of fresh NMM supplemented with 0.5 mM azidohomoalanine (Aha). After incubation at 37 ˚C for 30 min, expression of GST-TCS-Aha75Ub was induced by addition of 1 mM IPTG. Cells were grown at 25 ˚C overnight reaching an OD_{600} of approximately 2.7. Then, cells were pelleted, resuspended in 1xPBS buffer containing 1% Triton X-100, and lysed by sonication. The lysate was clarified by high speed centrifugation. The supernatant was transferred to a new tube and incubated with glutathione agarose beads at 4 ˚C for 5 h. The beads were packed into a column, washed three times with 1x PBS, and incubated with 10 units of thrombin at room temperature overnight. The released Aha75Ub was eluted with small volumes (9x500 µL) of 1x PBS. All the elution fractions were analyzed by SDS-PAGE and quantified by BCA assay.

Generation of branched Ub oligomers

15 µM C_6C_{11}Ub-PA, $C_{11}C_{48}$Ub-PA or $C_{11}C_{63}$Ub-PA were mixed with 2.5 eq. Aha75Ub, and 15 µM $C_6C_{11}C_{48}$Ub-PA was mixed with 3.5 eq. Aha75Ub in 20 mM Tris-HCl (pH 7.0). 0.5 mM SDS and 5 mM THPTA (final conc.) were added, followed by argon flushing. Click reaction was initiated by 2.5 mM of $Cu(MeCN)_4BF_4$, and samples were incubated on ice for 1 h. All the samples were analyzed by SDS-PAGE followed by Commassie blue staining and determination of the yield of the branched Ub oligomers by quantifying the intensity of the respective bands using the ImageJ software (NIH, Bethesda, MD, USA).

Western blot analysis

SDS-PAGE resolved proteins were transferred to nitrocellulose membrane. After blocking with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), the membrane was probed with 1 µg/mL K48-linkage specific antibody (Millipore), and further probed with an HRP-conjugated secondary antibody (Jackson ImmunoResearch). Finally, antibody-decorated proteins were visualized by chemiluminescence (LAS-3000, Fujifilm).

Size exclusion chromatography

Up-scaled click reaction containing 0.5 mg of $C_{11}C_{48}$Ub-PA and 1.25 mg of Aha75Ub was performed as described above. The reaction sample was applied directly onto the column (HiLoad 16/600 Superdex 75 PG) via a capillary loop (2 mL) using the ÄKTA purifier FPLC system. 25 mM Tris-HCl (pH 7.5) supplemented with 300 mM NaCl was used as the elution buffer. The elution speed was set at 0.5 mL/min and the size of collected fractions

at 1 mL per tube. The elution fractions were analyzed by SDS-PAGE followed by Coomassie blue staining. The isolated branched trimer was concentrated by Amicon Ultra Centrifugal Filter (10 kDa MWCO) and quantified by BCA protein assay.

Mass spectrometry analysis

Ub_2-$^{11/48}$Ub was digested by trypsin (Promega) and desalted by U-C18 Zip Tip (Merck Millipore). Tryptic peptides were measured by LC-MS/MS, and the data was analyzed by MaxQuant.

Stability assay

3.5 μM wild-type K48-linked trimer $Ub_{3\text{-}wt}^{48}$ (BostonBiochem) or the branched trimer Ub_2-$^{11/48}$Ub were incubated with 50 nM Isopeptidase (USP5, human recombinant; BostonBiochem) or 1 μM His6-Otubain-1, isoform 1 (OTUB1; BostonBiochem) at 37 ^{o}C in DUB-reaction buffer (50 mM Tris-HCl, pH 7.6, supplemented with 25 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT). At 1 min and 60 min, samples were withdrawn, mixed with 6x loading buffer, and heated at 95 ^{o}C. All the samples were analyzed by SDS-PAGE followed by Coomassie blue staining.

HEK293T cells were lysed in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl, 1 mM DTT, and 1% NP40) and incubated on ice for 20 min. The lysates were centrifuged at 15000 rpm at 4 ^{o}C for 10 min. The supernatant was transferred to a new tube. 3.5 μM $Ub_{3\text{-}wt}^{48}$ or Ub_2-$^{11/48}$Ub were incubated with 2.8 mg/mL cell lysate at 37 ^{o}C in DUB-reaction buffer. At 1 min and 60 min, samples were withdrawn, mixed with 6x loading buffer, and heated at 95 ^{o}C. All the samples were analyzed by SDS-PAGE followed by Coomassie blue staining.