Electronic Supplementary Material (ESI) for ChemComm.

<Supporting Information>

Facile synthesis of covalent probes to capture enzymatic intermediates during E1 enzyme catalysis

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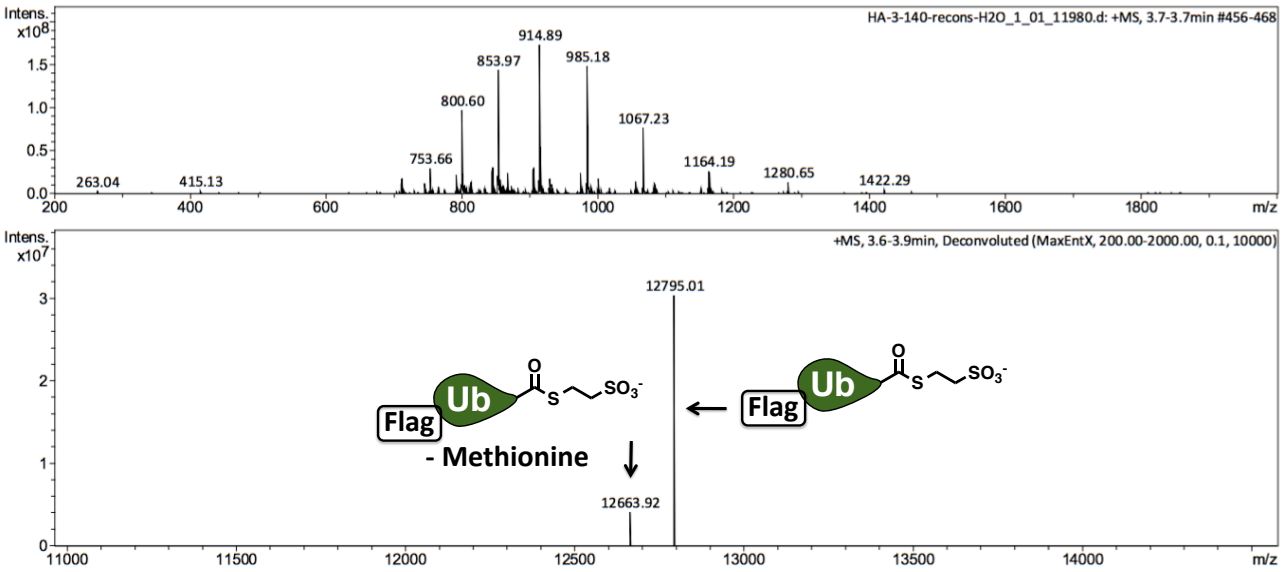


Figure S1. Intact protein mass-spectrometry of Flag-tagged Ub~Mes prepared by intein chemistry. ~10% of N-terminal methionine excision product was formed during the protein expression in *E. coli* based on the ionization intensity.

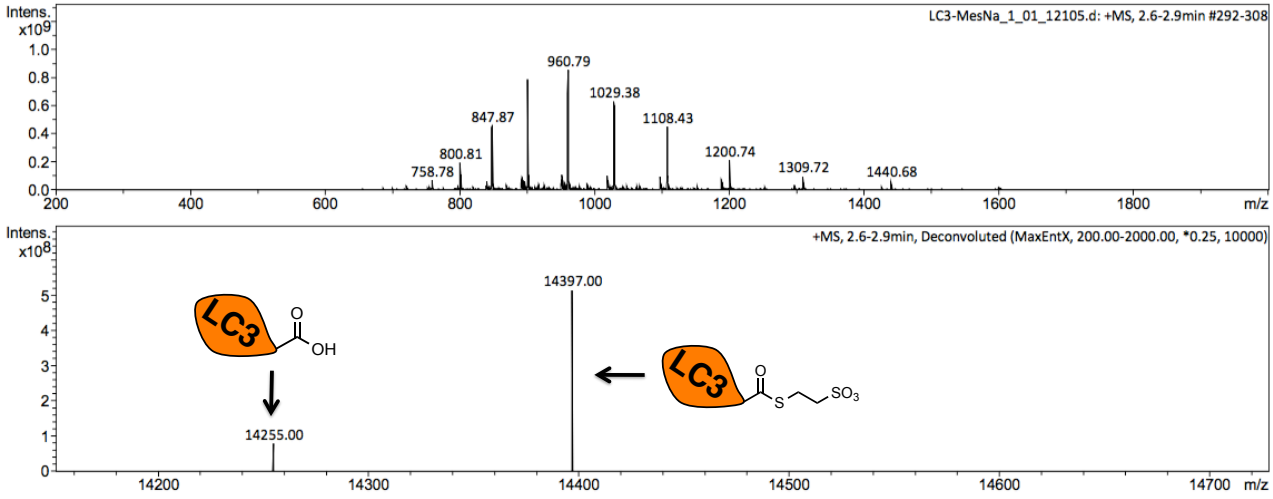


Figure S2. Intact protein mass-spectrometry of LC3~Mes prepared by the E1-mediated chemoenzymatic synthesis.

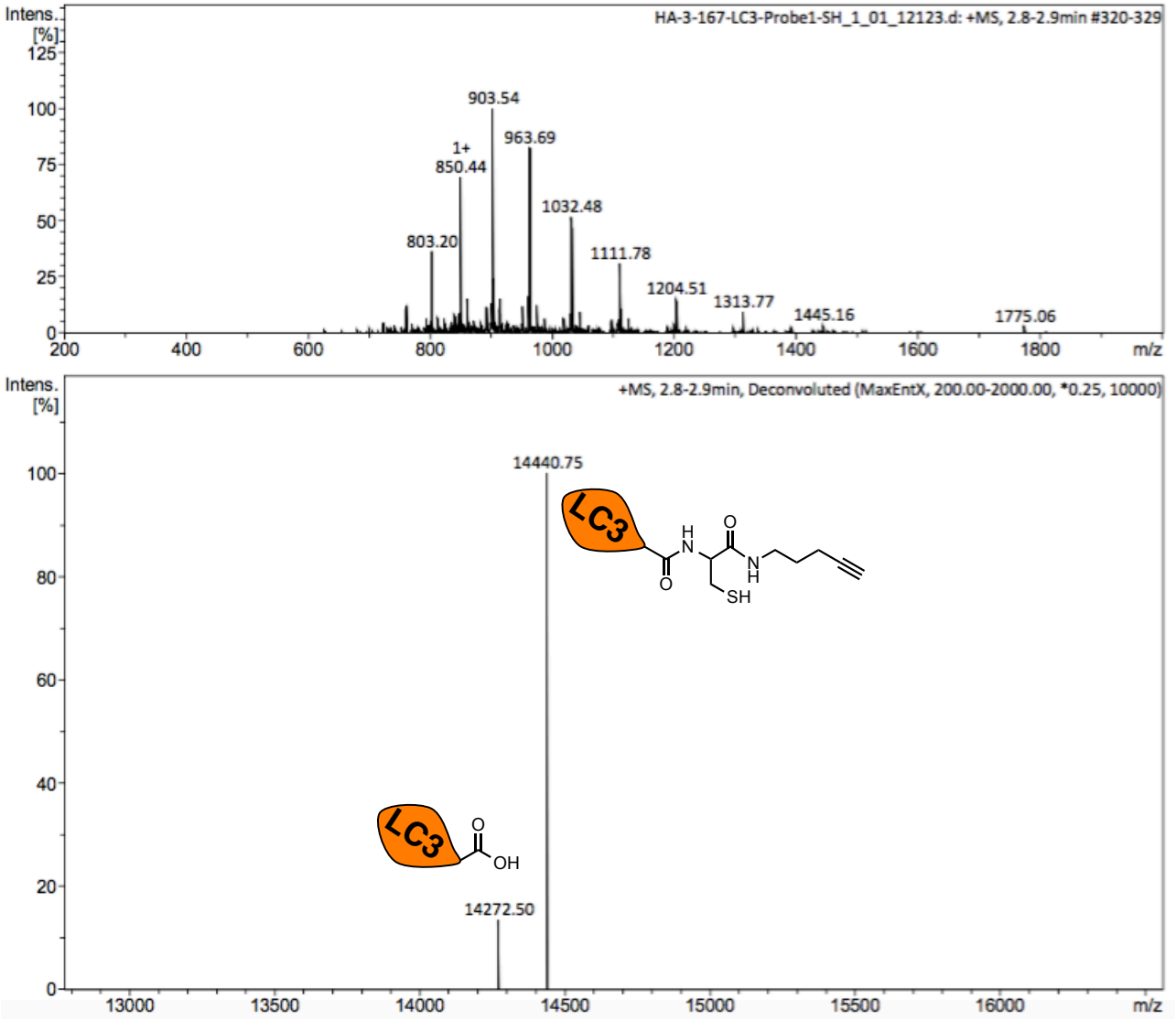


Figure S3. Intact protein mass-spectrometry of LC3-Probe1-SH intermediate after the native chemical ligation reaction.

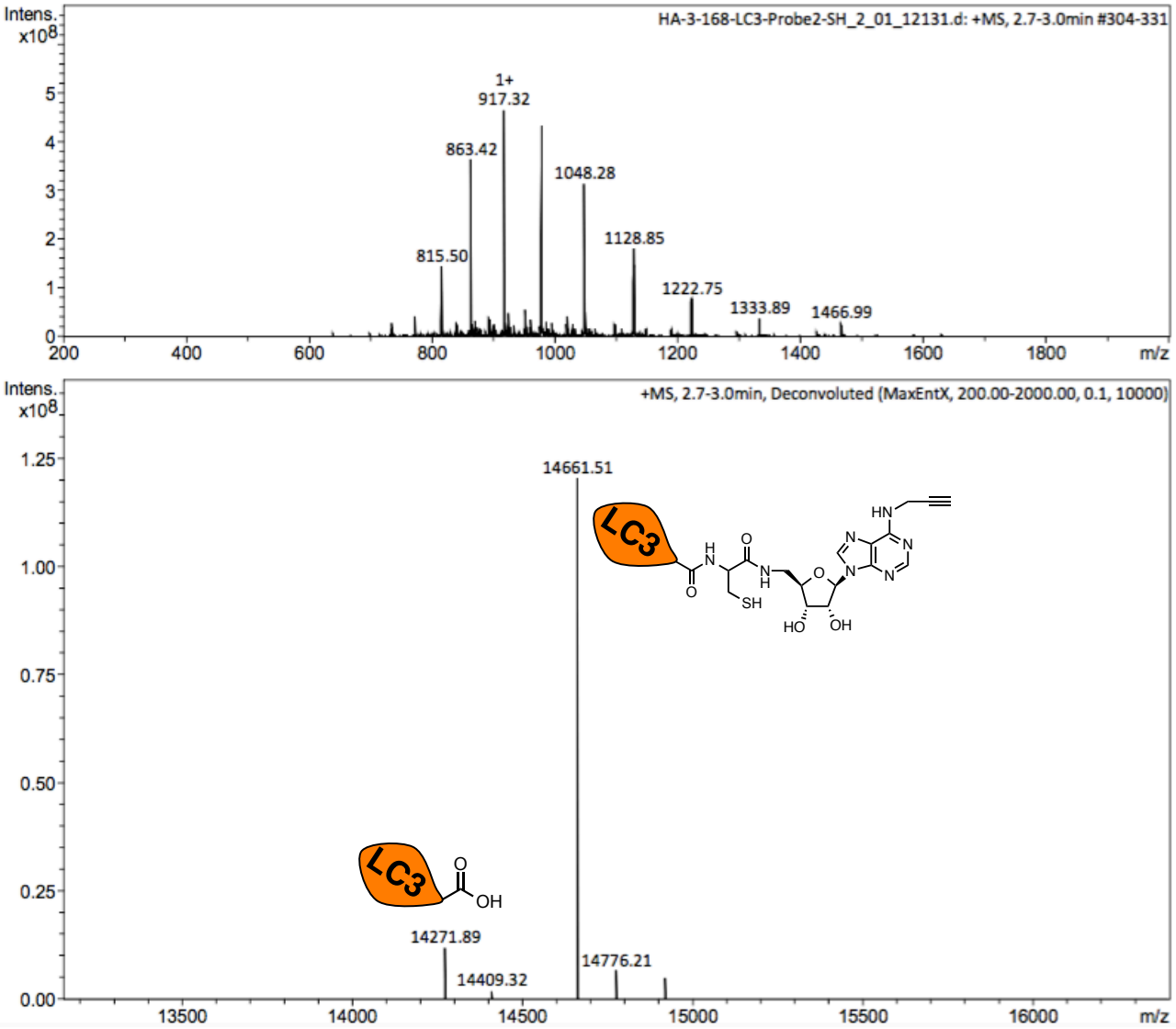


Figure S4. Intact protein mass-spectrometry analysis of LC3-Probe2-SH intermediate after the native chemical ligation reaction.

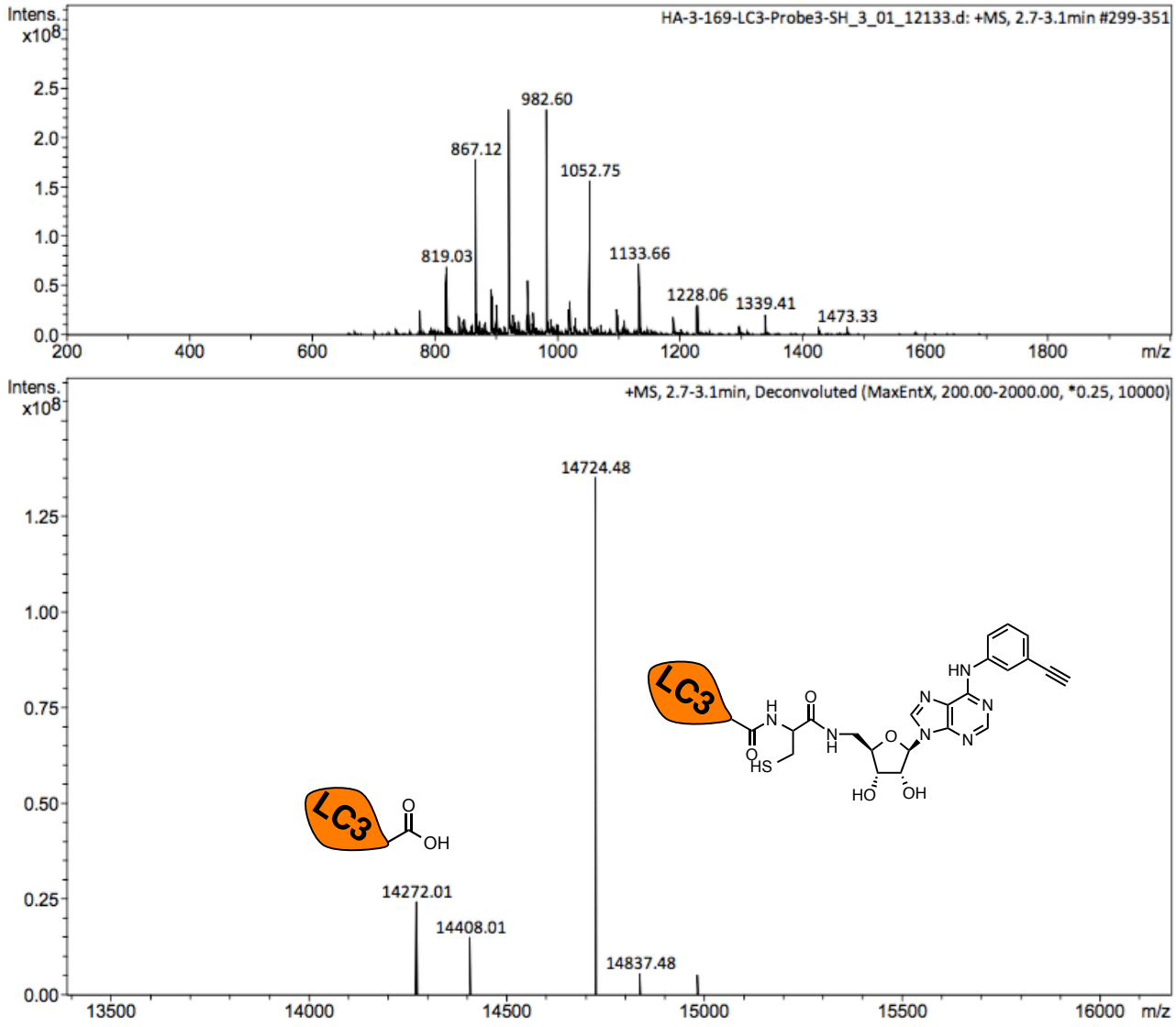


Figure S5. Intact protein mass-spectrometry analysis of LC3-Probe3-SH intermediate after the native chemical ligation reaction.

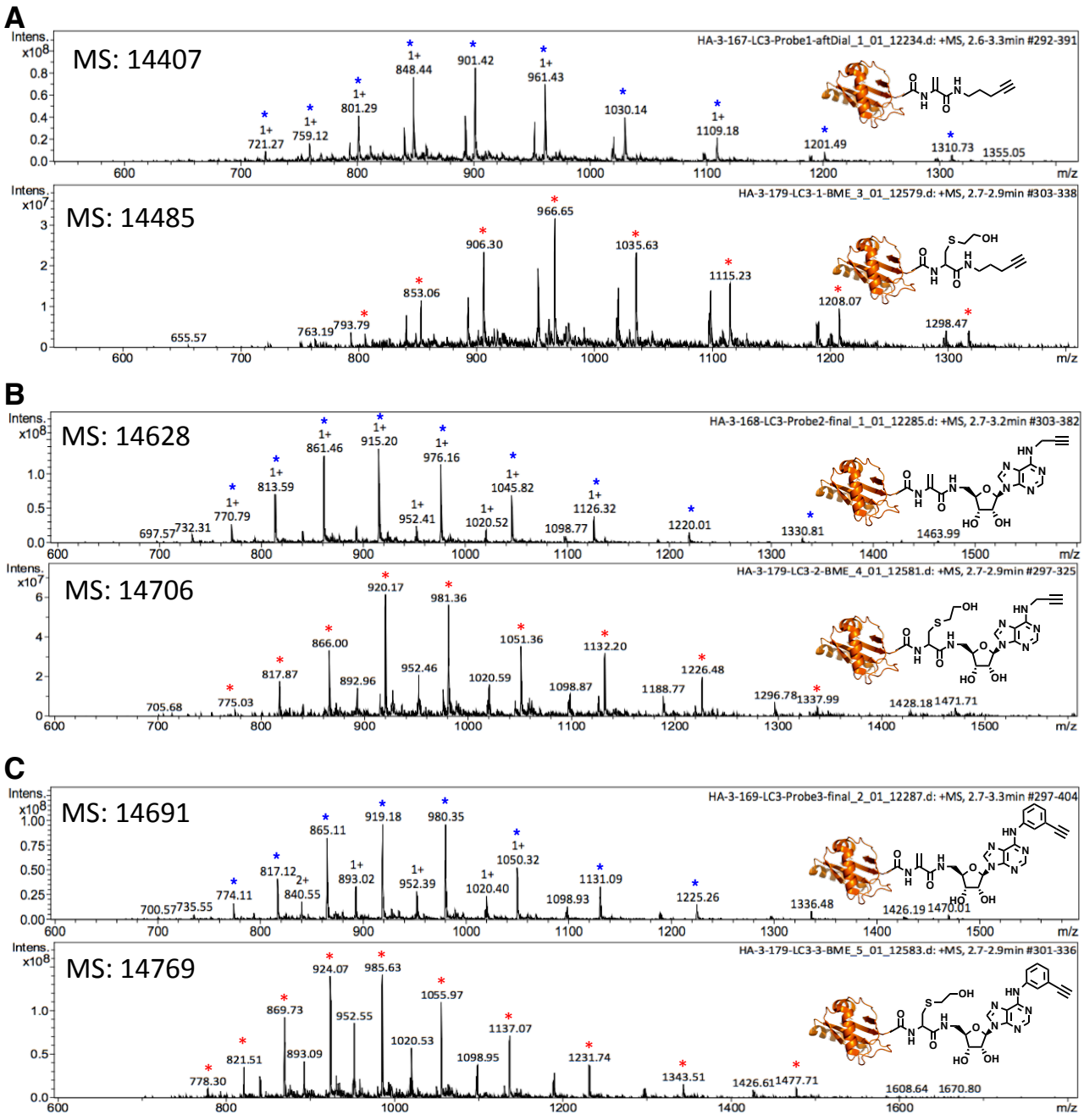


Figure S6. Treatment of LC3-Probes1-3 (30 μM) with β-mercaptoethanol (1 M) in pH 8.0 buffer for 2 hours at room temperature resulted in complete conversion of the probes into the Michael reaction products.

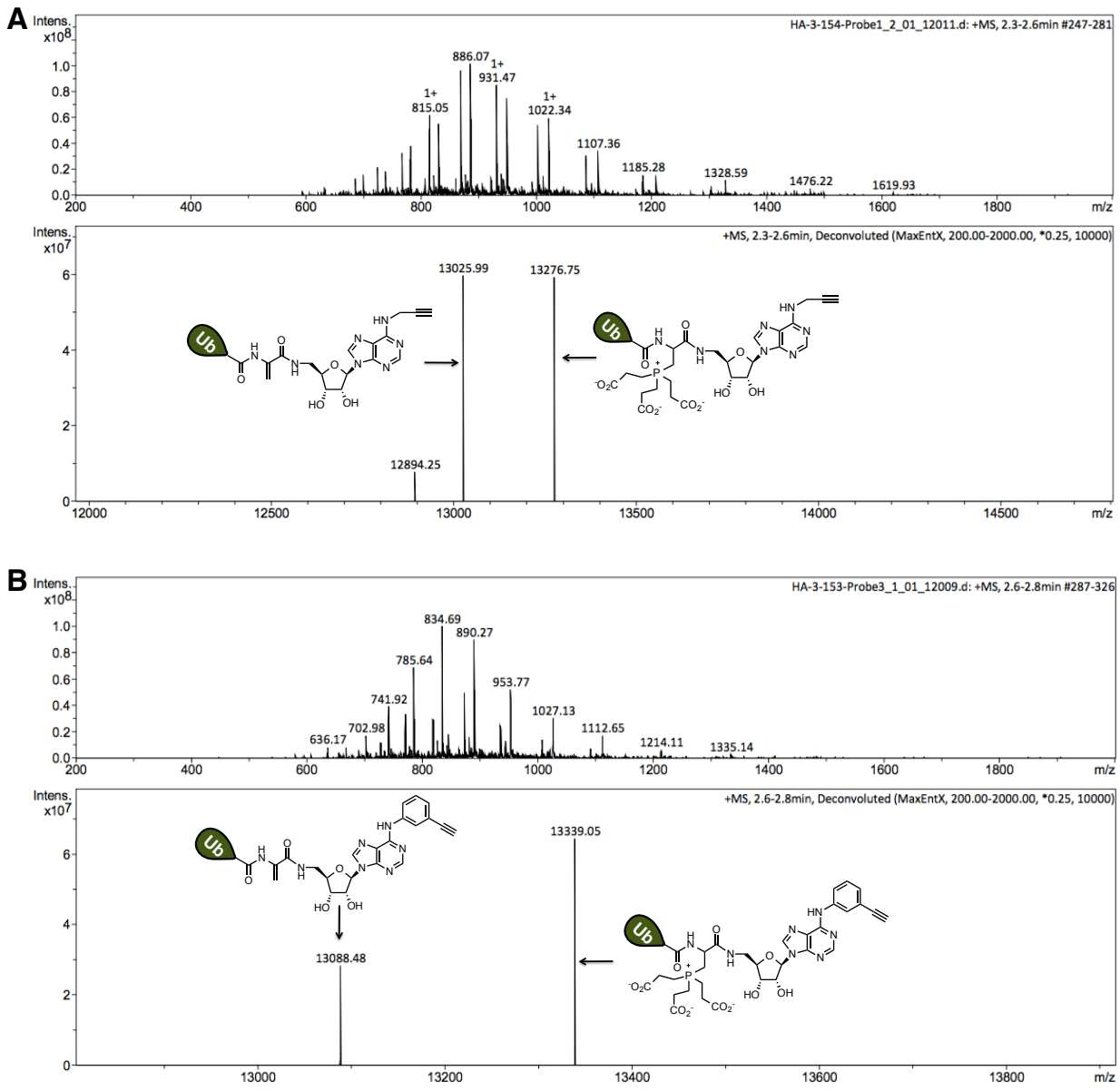


Figure S7. Treatment of Ub-Probes (50 μM) with TCEP (1 mM) at 37 °C for 1 hour resulted in the formation of Michael reaction products. Since TCEP can react with UBL-Probes**1-3**, caution is required when using TCEP as a reducing agent.

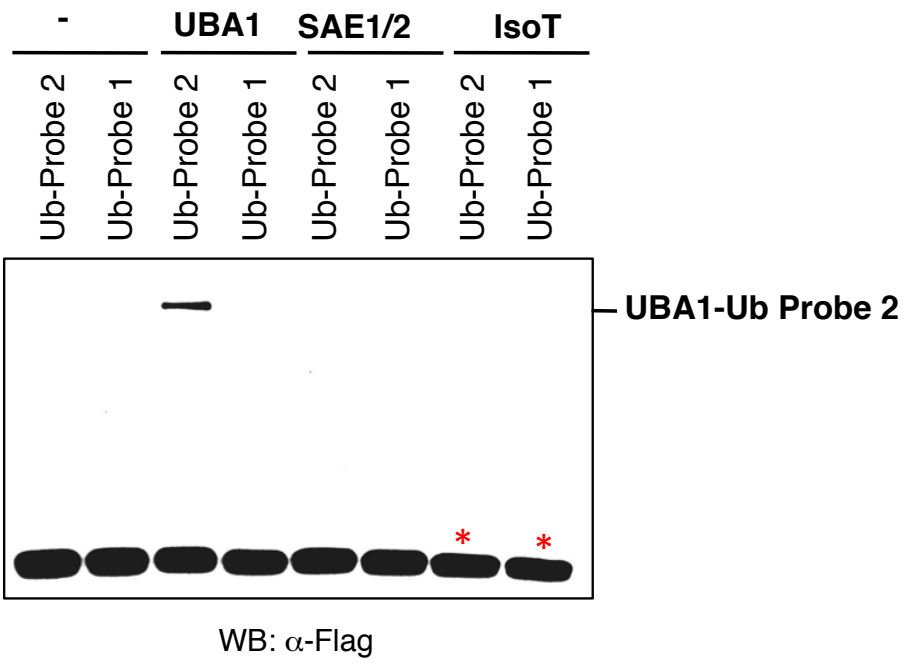
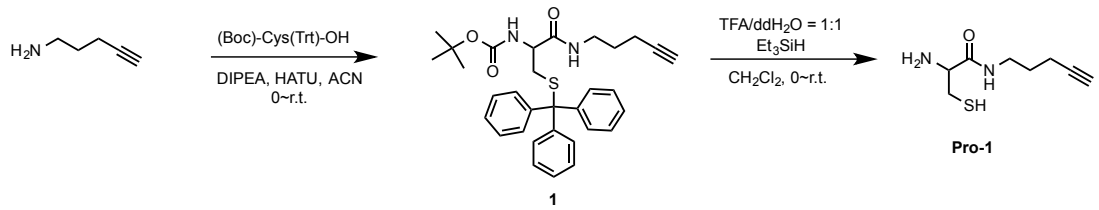


Figure S8. Labelling of UBA1 (2 μM), SUMO E1 (2 μM), and IsoT (2 μM) with Ub-Probe1-2 (12 μM) was visualized by Western Blotting. We detected the formation of UBA1-Ub-Probe2 covalent adduct. However, the cleavage of the free probes by IsoT DUB (red stars) was not discernable in this WB image, highlighting the advantage of the alkyne tag-mediated click chemistry method to detect and quantify free Ub-Probe1-3 and their covalent adducts with E1 enzyme.

II. Synthesis

General information.

Methanol (ACS grade), ethyl acetate (ACS grade, and HPLC grade), hexane (ACS grade), acetonitrile (ACS grade), chloroform (ACS grade) and acetone (ACS grade) were purchased from Fisher Scientific and used without further purification. Dichloromethane and toluene were purified by passing over activated alumina. Commercially available reagents were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on pre-coated glass-backed plates (60 Å silica gel, 0.25mm, Whatman), and components were visualized by UV light (254 and 365 nm) or by treating the plates with anisaldehyde, $KMnO_4$, and ninhydrin stains followed by heating. Flash column chromatography was performed over ultra pure silica gel (230-400 mesh) from Silicycle. ^{1}H and ^{13}C NMR spectra were obtained on Bruker AVANCE III 500 MHz spectrometers. Chemical shifts were reported in ppm relative to the residual solvent peak ($CDCl_3$, ^{13}C 77.00; TMS: 0.00). Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublets); ddd (doublet of doublet of doublets); dt (doublets of triplets); td (triplet of doublets); brs (broad singlet). Coupling constants were reported in Hz. High resolution mass analysis of the final ABP analogs were conducted using LC-TOF: Agilent 6210A. Mass analysis of the other intermediates were conducted using LC-MS: Bruker AmaZon SL Ion Trap and LC-MS: Bruker AmaZon X Ion Trap.



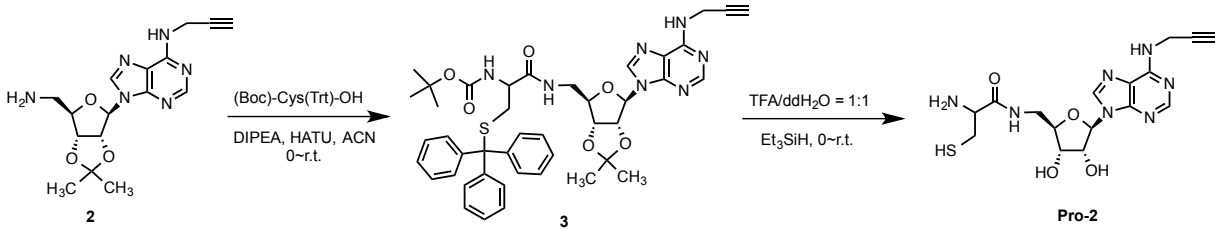
Synthesis of 1

Round bottom flask (25 ml) was charged with Boc-Cys(Trt)-OH (500.0 mg, 1.08 mmol, 1 equiv.) and 10 ml acetonitrile, followed by addition of HATU (490.5 mg, 1.29 mmol, 1.2 equiv), pent-4-yn-1-amine (107.5 mg, 1.29 mmol, 1.2 equiv.), and diisopropylethylamine (206.5 μl, 1.19 mmol, 1.1 equiv.) at 0 °C. The reaction mixture was then stirred at r.t. for 2 hours. Acetonitrile was evaporated under the reduced pressure, and the reaction mixture was redissolved in CH_2Cl_2 and saturated NH_4Cl. Reaction product was extracted three times with CH_2Cl_2, and the gathered organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (MeOH:CH_2Cl_2 gradient 0 → 7 % MeOH) to provide 1 (319 mg, 59%) as white solid. TLC R_f = 0.45 (CH_2Cl_2 only); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.39 – 7.35 (m, 6H), 7.24 (dd, *J* = 8.5, 6.8 Hz, 6H), 7.19 – 7.15 (m, 3H), 6.12 (t, *J* = 5.8 Hz, 1H), 4.74 (s, 1H), 3.84 – 3.73 (m, 1H), 3.25 (q, *J* = 6.5 Hz, 2H), 2.66 (dd, *J* = 12.6, 6.9 Hz, 1H), 2.46 (dd, *J* = 12.8, 5.4 Hz, 1H), 2.14 (td, *J* = 7.0, 2.7 Hz, 2H), 1.90 (t, *J* = 2.7 Hz, 1H), 1.64 (q, *J* = 6.7 Hz, 2H), 1.36 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 170.47, 144.41, 129.58, 128.07, 126.90, 83.36, 80.31, 69.22, 67.22, 53.64, 38.52, 33.82, 29.72, 28.30, 27.91, 15.95. MS calcd for $C_{32}H_{36}N_2O_3S$ $[M + Na]^+$: 551.24; Found: *m/z* 551.27

Synthesis of Pro-1

A flask containing compound **1** (100 mg, 0.189 mmol, 1 equiv.) in CH_2Cl_2 (0.8 mL) was added with a solution of TFA in ddH_2O (1:1 v/v, total volume 1 mL) at 0 ºC. Then, 0.2 mL of Et_3SiH

(10%) was added to the mixture to scavenge trityl cation. The resulting reaction mixture was stirred at r.t. for 1hr, while the reaction progress was monitored by thin layer chromatography. Subsequently, CH_2Cl_2, TFA, Et_3SiH and ddH_2O were removed under the flow of N_2 gas for 2 hours. The remaining residue was purified with silica gel flash column chromatography (MeOH : CH_2Cl_2 gradient 0 → 10 % MeOH) to provide Pro-1 (quantitative yield) as transparent liquid. TLC R_f = 0.25 (CH_2Cl_2 : MeOH = 10 : 1); ^{1}H NMR (500 MHz, Methanol-d_4) δ 3.99 (dd, J = 6.8, 5.3 Hz, 1H), 3.42 - 3.30 (m, 3H), 3.09 - 2.93 (m, 2H), 2.29 (d, J = 5.5 Hz, 2H), 1.82 - 1.73 (m, 2H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 166.97, 82.52, 68.84, 54.76, 38.34, 27.76, 24.85, 15.20. MS calcd for $C_8H_{14}N_2OS$ $[M + H]^+$: 187.08; Found: m/z 187.07.



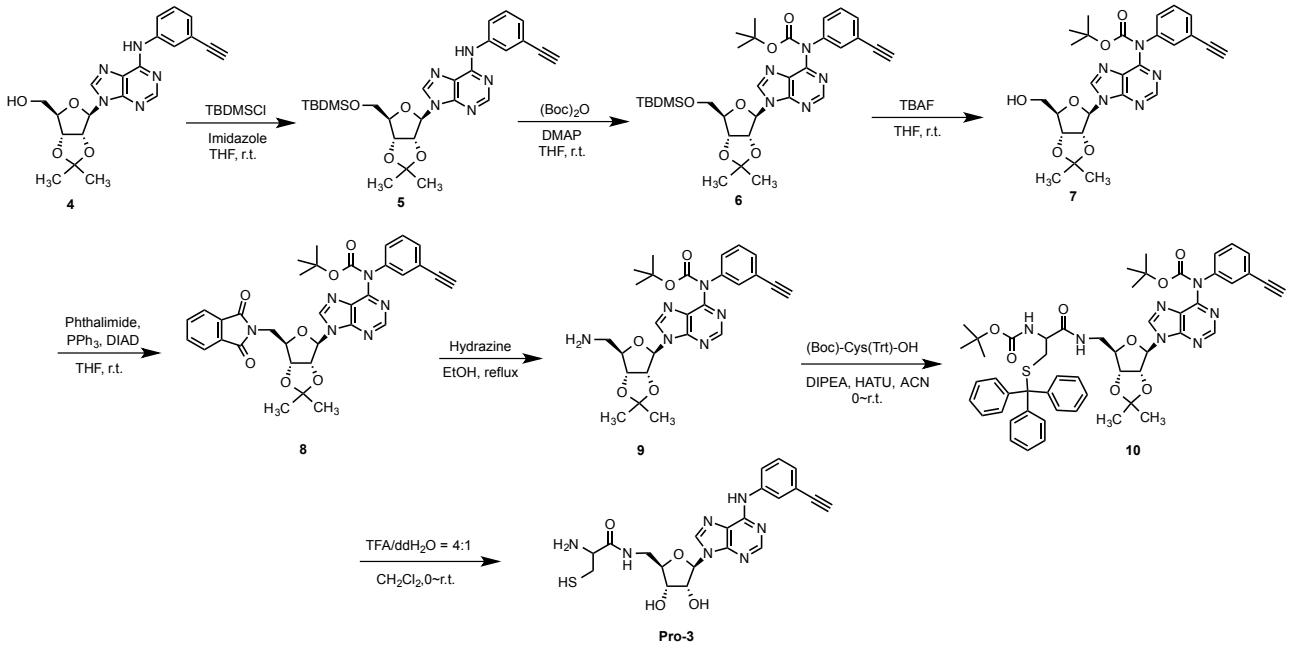
Synthesis of 3

Compound 2 was prepared following the previously reported procedure.[1] To a flask containing Boc-Cys(Trt)-OH (44.5 mg, 0.096 mmol, 1.1 equiv.) and ACN (1.5 ml), was added HATU (39.7mg, 0.104 mmol, 1.2 equiv.) at 0 °C, followed by the addition of compound **2** (30 mg, 0.087 mmol) and diisopropylethylamine (16.7 μl, 0.096 mmol, 1.1 equiv.). The reaction was completed in 30 min when monitored by TLC. White precipitate was filtered and the filtrate was concentrated under reduced pressure. Then, the reaction mixture was redissolved in CH_2Cl_2 and saturated NH_4Cl solution. The reaction product was extracted three times with CH_2Cl_2. Collected organic extracts were dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (MeOH : CH_2Cl_2 gradient 0 → 10 % MeOH) to provide **3** (91.1 mg, 99%) as white solid. TLC R_f = 0.73 (MeOH:CH_2Cl_2 =

1:10); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.98 – 8.88 (m, 1H), 8.27 (s, 1H), 7.76 (s, 1H), 7.31 – 7.24 (m, 6H), 7.02 (t, *J* = 7.7 Hz, 6H), 6.92 (t, *J* = 7.3 Hz, 3H), 6.33 – 6.17 (m, 1H), 5.65 – 5.59 (m, 2H), 4.90 – 4.79 (m, 1H), 4.58 (d, *J* = 5.9 Hz, 1H), 4.42 (brs, 2H), 4.26 – 4.13 (m, 1H), 3.09 (d, *J* = 14.5 Hz, 1H), 2.55 – 2.38 (m, 2H), 2.23 (t, *J* = 2.5 Hz, 1H), 1.52 (s, 3H), 1.39 (s, 12H). ^{13}C NMR (126 MHz, CDCl3) δ 170.85, 165.76, 155.03, 152.81, 144.19, 140.06, 129.31, 127.76, 126.48, 114.48, 93.49, 82.74, 81.62, 79.88, 71.65, 66.55, 52.80, 41.35, 38.63, 34.83, 28.46, 27.50, 24.76. MS calcd for $C_{43}H_{47}N_7O_6S$ $[M + H]^+$: 790.33; Found: m/z 790.22.

Synthesis of Pro-2

Compound **3** (93 mg, 0.116 mmol, 1 equiv.) in a 20 ml vial was treated with a solution of TFA in ddH_2O (1:1 v/v, total volume 8 mL) at 0 ºC. Then, 74 µL of Et_3SiH (0.464 mmol, 4 equiv.) was added to the mixture to scavenge trityl cation, and the reaction mixture was stirred at r.t. for 1 hour. The reaction progress was monitored by thin layer chromatography. CH_2Cl_2, TFA, Et_3SiH and ddH_2O were removed under the stream of N_2 gas for 2 hours. The remaining residue was purified by reverse phase high performance liquid chromatography (solvent A: 95% ddH_2O, 5% acetonitrile, 0.1% TFA, solvent B: 95% acetonitrile, 5% ddH_2O, 0.1% TFA. Gradient method: 0 to 100% of solvent B for 20 minutes). Collected fractions were lyophilized to yield **Pro-2** (quantitative yield). TLC R_f = 0.25 (CH_2Cl_2 : MeOH = 10 : 1); ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.43 (s, 2H), 6.02 (d, J = 5.5 Hz, 1H), 4.84 (t, J = 5.5 Hz, 1H), 4.54 – 4.35 (brs, 2H), 4.27 (dd, J = 5.4, 4.0 Hz, 1H), 4.21 (dt, J = 7.9, 4.1 Hz, 1H), 4.07 (t, J = 5.9 Hz, 1H), 3.84 (dd, J = 14.0, 7.0 Hz, 1H), 3.59 (dd, J = 14.2, 4.0 Hz, 1H), 3.01 (qd, J = 14.6, 5.9 Hz, 2H), 2.76 (s, 1H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 168.72, 162.20, 161.91, 142.87, 118.78, 116.47, 90.80, 84.63, 79.89, 74.91, 72.93, 56.20, 42.94, 42.82, 31.2, 26.32. HRMS calcd for $C_{16}H_{21}N_7O_4S$ $[M + H]^+$: 408.14; Found: m/z 408.1448.



Synthesis of 5

Compound **4** was prepared via the previously reported procedure.[1] Compound **4** (100 mg, 0.245 mmol, 1 equiv.) dissolved in THF (2.5 ml) was treated with tert-Butyldimethylsilyl chloride (55.4 mg, 0.367 mmol, 1.5 equiv.) and imidazole (50 mg, 0.735 mmol, 3 equiv.). The reaction mixture was stirred at r.t. for 2 hours. Upon the completion of the reaction as judged by TLC, white precipitate was filtered. The filtrate was concentrated under reduced pressure and redissolved in CH_2Cl_2 and saturated NH_4Cl. Aqueous layer was extracted with CH_2Cl_2 for three times. The resulting organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (EtOAc : Hexane = 1:5 → 1:2.5) to provide compound **5** (105.3 mg, 82%) as white solid. TLC R_f = 0.23 (EtOAc : Hexane = 1:3); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.52 (s, 1H), 8.05 (s, 1H), 7.97 (d, *J* = 1.9 Hz, 1H), 7.77 - 7.73 (m, 1H), 7.72 (s, 1H), 7.27 (t, *J* = 7.9 Hz, 1H), 7.20 - 7.15 (m, 1H), 6.15 (d, *J* = 2.4 Hz, 1H), 5.25 (dd, *J* = 6.2, 2.5 Hz, 1H), 4.91 (dd, *J* = 6.2, 2.4 Hz, 1H), 4.40 (d, *J* = 2.5 Hz, 1H), 3.84 (dd, *J* = 11.2, 3.9 Hz, 1H), 3.72 (dd, *J* = 11.2, 4.2 Hz, 1H), 3.04 (s, 1H), 2.04 (s, 1H), 1.59 (s, 3H), 1.36 (s, 3H), 0.79 (s, 9H), -0.03 (d, *J* = 1.8 Hz, 6H). ^{13}C NMR (126 MHz,

Chloroform-*d*) δ 152.93, 151.95, 149.07, 139.56, 138.78, 129.02, 127.16, 123.45, 122.83, 120.87, 120.64, 114.12, 91.77, 87.50, 85.09, 83.52, 81.53, 63.58, 27.25, 25.83, 25.41, 18.34, -5.43, -5.51. MS calcd for $C_{27}H_{35}N_5O_4Si$ $[M + H]^+$: 522.25; Found: m/z 522.30.

Synthesis of 6

Round bottom flask (10 mL) containing compound **5** (100 mg, 0.19 mmol, 1 equiv.) dissolved in 1 ml THF, was added with di-tert-butyl dicarbonate (63.5 mg, 0.29 mmol, 1.5 equiv.) and DMAP (1.2 mg, 5%). The reaction mixture was stirred for 2 hours at r.t. When complete conversion of compound **5** was confirmed based on the thin layer chromatography, THF was evaporated and the remaining residue was redissolved in ddH_2O and CH_2Cl_2. The organic compound was extracted with CH_2Cl_2 for two times. The resulting organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (EtOAc : Hexane = 1:10 → 1:3, gradient) to provide compound **6** (111.6 mg, 94%) as white solid. TLC R_f = 0.3 (EtOAc : Hexane = 1:3); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.72 (s, 1H), 8.25 (s, 1H), 7.35 – 7.30 (m, 1H), 7.29 (q, J = 1.6 Hz, 1H), 7.28 – 7.25 (m, 2H), 6.18 (d, J = 2.6 Hz, 1H), 5.22 (dd, J = 6.1, 2.6 Hz, 1H), 4.91 (dd, J = 6.2, 2.5 Hz, 1H), 4.40 (td, J = 3.8, 2.4 Hz, 1H), 3.83 (dd, J = 11.3, 3.7 Hz, 1H), 3.73 (dd, J = 11.3, 4.1 Hz, 1H), 2.97 (s, 1H), 1.59 (s, 3H), 1.40 (s, 9H), 1.36 (s, 3H), 0.78 (s, 9H), -0.05 (d, J = 6.8 Hz, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 153.02, 152.60, 152.52, 152.39, 142.63, 140.99, 131.22, 130.55, 128.90, 128.73, 128.50, 122.95, 114.25, 91.68, 87.37, 85.01, 82.92, 81.43, 63.56, 27.93, 27.26, 25.88, 25.38, 18.34, -5.43, -5.51. MS calcd for $C_{32}H_{43}N_5O_6Si$ $[M + H]^+$: 622.30; Found: m/z 622.33.

Synthesis of 7

A round bottom flask (10 mL) containing compound **6** (110 mg, 0.176 mmol, 1 equiv.) in 1 ml THF, was charged with tetra-n-butylammonium fluoride solution (214 µl of 1M THF solution, 0.215 mmol, 1.2 equiv.) at 0 °C. The reaction mixture was stirred for 1 hour, followed by evaporation of THF. The remaining residue was redissolved in CH_2Cl_2 and saturated NaCl solution. The organic compound was extracted with CH_2Cl_2 3x10 mL, after which the organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (EtOAc : Hexane = 1:3 → 2:1, gradient) to provide compound **7** (88.9 mg, 99%) as white solid. TLC R_f = 0.5 (EtOAc : Hexane = 2:1); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.65 (s, 1H), 8.05 (s, 1H), 7.35 (dt, J = 7.4, 1.4 Hz, 1H), 7.31 – 7.27 (m, 2H), 7.24 (dt, J = 8.1, 1.4 Hz, 1H), 5.89 (d, J = 4.8 Hz, 1H), 5.45 (d, J = 11.0 Hz, 1H), 5.19 (dd, J = 6.0, 4.8 Hz, 1H), 5.07 (dd, J = 6.0, 1.4 Hz, 1H), 4.49 (q, J = 1.6 Hz, 1H), 3.92 (dd, J = 12.6, 1.7 Hz, 1H), 3.76 (td, J = 10.3, 5.3 Hz, 1H), 3.00 (s, 1H), 1.60 (s, 3H), 1.42 (s, 9H), 1.33 (s, 3H).^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.16, 153.91, 152.46, 151.79, 151.65, 143.44, 140.78, 131.39, 130.83, 129.43, 129.00, 128.59, 123.06, 114.29, 94.12, 86.11, 83.21, 82.97, 82.80, 81.54, 77.88, 77.29, 77.04, 76.78, 63.34, 60.40, 27.98, 27.62, 25.23, 21.06, 14.21. MS calcd for $C_{26}H_{29}N_5O_6$ $[M + H]^+$: 508.21; Found: m/z 508.23.

Synthesis of 8

A 5 ml round bottom flask containing compound **7** (40 mg, 0.078 mmol, 1 equiv.) and phthalimide (14 mg, 0.094 mmol, 1.2 equiv.) in 1 ml THF was treated with triphenylphophine (30 mg, 0.108 mmol, 1.5 equiv.) at 0 °C. Subsequently, diisopropyl azodicarboxylate (26 µl, 0.132 mmol, 1.7 equiv.) in 1 ml THF was added dropwise. The resulting reaction mixture was stirred at 0

°C for 1 hour, and the reaction progress was monitored by TLC. Subsequently, THF was concentrated under the reduced pressure, and the remaining residue was redissolved in CH_2Cl_2 and ddH_2O, followed by extraction with CH_2Cl_2 for two times. Organic layer was dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (EtOAc : Hexane = 1:5 → 1:1, gradient) to provide compound **8** as white crystals. TLC R_f = 0.35 (EtOAc : Hexane = 1:1); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.47 (s, 1H), 8.06 (s, 1H), 7.70 (dt, J = 7.0, 3.5 Hz, 2H), 7.62 (dd, J = 5.5, 3.0 Hz, 2H), 7.36 – 7.30 (m, 2H), 7.28 (dd, J = 4.9, 2.4 Hz, 2H), 6.06 (d, J = 1.9 Hz, 1H), 5.54 (dd, J = 6.3, 1.9 Hz, 1H), 5.18 (dd, J = 6.4, 3.3 Hz, 1H), 4.54 (td, J = 6.5, 3.4 Hz, 1H), 3.92 (d, J = 6.5 Hz, 2H), 2.95 (s, 1H), 1.53 (s, 3H), 1.42 (s, 9H), 1.33 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.13, 167.97, 153.25, 152.55, 152.29, 152.16, 143.72, 141.02, 134.06, 131.84, 131.20, 130.51, 129.05, 128.87, 128.48, 123.35, 122.90, 114.62, 90.88, 84.84, 84.08, 82.98, 82.93, 82.53, 77.70, 77.33, 77.27, 77.07, 76.82, 60.39, 39.44, 29.69, 28.00, 27.06, 25.36, 21.96, 21.86, 21.78, 21.69, 21.05, 14.21. MS calcd for $C_{34}H_{32}N_6O_7[M + H]^+$: 637.23; Found: m/z 637.24.

Synthesis of 9

A 5 ml round bottom flask containing compound **8** (49.6 mg, 0.078 mmol, 1 equiv.) was treated with 360 μl EtOH and N_2H_4 (49 μl, 1.56 mmol, 20 equiv.) and stirred at 70 °C for 30 min. White precipitate was filtered, and the filtrate was concentrated under the reduced pressure. The resulting residue was redissolved in CH_2Cl_2 and saturated NH_4Cl solution. The reaction product was extracted by CH_2Cl_2 for three times. Organic layers were dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified using silica gel flash column chromatography (CH_2Cl_2 : MeOH = 30:1 → 20:1, gradient) to provide compound **9**. TLC R_f

= 0.45 (CH_2Cl_2 : MeOH = 1:1); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.68 (d, J = 2.0 Hz, 1H), 8.14 (s, 1H), 7.36 – 7.22 (m, 4H), 6.06 (d, J = 3.1 Hz, 1H), 5.38 (dd, J = 6.5, 3.0 Hz, 1H), 4.96 (dd, J = 6.5, 3.6 Hz, 1H), 4.27 – 4.19 (m, 1H), 2.97 (d, J = 18.1 Hz, 3H), 2.10 – 2.03 (m, 2H), 1.58 (s, 3H), 1.41 (s, 9H), 1.34 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.16, 153.29, 152.42, 152.38, 143.15, 140.92, 131.34, 130.69, 128.94, 128.72, 128.58, 122.97, 114.84, 114.58, 90.73, 87.41, 87.35, 83.76, 82.98, 82.87, 81.75, 77.77, 77.29, 77.24, 77.03, 77.00, 76.78, 60.41, 43.69, 28.02, 28.00, 27.28, 25.38, 21.07, 14.21. MS calcd for $C_{26}H_{30}N_6O_5$ $[M + H]^+$: 507.23; Found: m/z 507.23.

Synthesis of 10

To a vial containing Boc-Cys(Trt)-OH (50.5 mg, 0.109 mmol, 1.1 equiv.) and 10 ml acetonitrile, was added HATU (45 mg, 0.118 mmol, 1.2 equiv), compound **9** (50 mg, 0.99 mmol, 1 equiv.), and diisopropylethylamine (19 μl, 0.109 mmol, 1.1 equiv.) at 0 °C. The reaction mixture was then stirred at r.t. for 2 hours. Acetonitrile was evaporated under the reduced pressure, and the reaction mixture was redissolved in CH_2Cl_2 and saturated NH_4Cl. The organic compound was extracted with CH_2Cl_2 for three times. Combined organic layers were dried over $MgSO_4$ and filtered. The filtrate was concentrated under the reduced pressure and the residue was purified with silica gel flash column chromatography (EtOAc : Hexane = 1:3 → 1.5:1, gradient) to provide compound **10** (49.5 mg, 53%) as a white solid. TLC R_f = 0.75 (EtOAc : Hexane = 2:1); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.65 (s, 1H), 8.14 – 8.06 (m, 1H), 8.02 (s, 1H), 7.40 – 7.34 (m, 2H), 7.29 (d, J = 7.5 Hz, 7H), 7.06 (d, J = 7.6 Hz, 6H), 6.97 (t, J = 7.3 Hz, 3H), 5.74 (d, J = 5.1 Hz, 1H), 5.29 (d, J = 7.7 Hz, 1H), 5.04 (t, J = 5.7 Hz, 1H), 4.73 – 4.64 (m, 1H), 4.41 (q, J = 2.4 Hz, 1H), 4.29 – 4.20 (m, 1H), 4.16 – 4.07 (m, 1H), 3.18 (d, J = 14.4 Hz, 1H), 3.00 (s, 1H), 2.59 – 2.42 (m, 2H), 1.52 (s, 3H),

1.44 (s, 9H), 1.35 (s, 9H), 0.99 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.16, 170.58, 155.02, 153.77, 152.56, 152.17, 151.75, 144.20, 143.51, 140.77, 131.57, 130.86, 129.34, 128.97, 128.80, 127.87, 126.61, 123.05, 114.78, 92.95, 83.15, 83.02, 82.83, 81.84, 81.55, 80.07, 77.91, 77.30, 77.25, 77.05, 77.00, 76.79, 66.80, 60.41, 53.24, 41.20, 34.48, 28.36, 28.02, 27.47, 24.91, 21.07, 14.22. MS calcd for $C_{53}H_{57}N_7O_8S$ $[M + H]^+$: 952.40; Found: m/z 952.40.

Synthesis of Pro-3

Compound **10** (49 mg, 0.052 mmol, 1 equiv.) in a 20 ml vial was treated with a solution of TFA in ddH_2O (1:1 v/v, total volume 8 mL) at 0 ºC. Then, 33 µL of Et_3SiH (0.206 mmol, 4 equiv.) was added to the reaction mixture to scavenge trityl cation. The reaction mixture was stirred at r.t. for 1 hour, and the reaction progress was monitored by thin layer chromatography. Subsequently, CH_2Cl_2, TFA, Et_3SiH and ddH_2O were removed under the flow of N_2 gas. The remaining residue was purified by reverse phase high performance liquid chromatography (solvent A: 95% ddH_2O, 5% acetonitrile, 0.1% TFA, solvent B: 95% acetonitrile, 5% ddH_2O, 0.1% TFA. Gradient method: 0 to 100% of solvent B for 20 minutes). Collected fractions were lyophilized to yield **Pro-3**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.10 (d, J = 11.8 Hz, 1H), 8.71 (q, J = 5.8 Hz, 1H), 8.60 (s, 1H), 8.51 (d, J = 5.0 Hz, 1H), 8.23 – 8.22 (m, 1H), 8.00 – 7.93 (m, 1H), 7.36 (t, J = 7.9 Hz, 1H), 7.17 (dt, J = 7.5, 1.3 Hz, 1H), 5.97 (dq, J = 5.8, 3.3, 2.7 Hz, 1H), 4.79 (ddd, J = 11.4, 8.3, 5.0 Hz, 1H), 4.21 – 4.12 (m, 2H), 4.10 – 3.90 (m, 3H), 3.19 (dd, J = 13.8, 5.3 Hz, 1H), 2.97 – 2.87 (m, 2H). MS calcd for $C_{21}H_{23}N_7O_4S$ $[M + H]^+$: 470.15; Found: m/z 470.06.

III. Bio-Protocols

General Information

Human recombinant SAE1/SAE2 and IsoT proteins were purchased from R&D Systems. Purchased proteins were used without further purification. In-gel fluorescence imaging was performed on a Typhoon 9600 (GE Healthcare). Proteins on polyacrylamide gels were visualized with Instant*Blue*™ (expedeon) staining solution.

Protein Expression

Expression of wild type UBA1 and C632A UBA1

6×His tagged mouse UBA1 plasmid was purchased from Addgene. The C632A mutant allele of UBA1 was generated using QuikChange mutagenesis (Agilent Technology). The His-tagged wtUBA1 and C632A UBA1 plasmids were transformed into BL21 (DE3) cells (Novagen). 1L LB madia containing 15μg/ml kanamycin was inoculated with 50 ml overnight culture and incubated at 37 °C until OD reached 0.6. Then, IPTG (0.5 mM final concentration) was added to the cell culture media at 16 °C, followed by 20 hours incubation at the same temperature. Cells were then harvested and lysed by sonication in cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM DTT, pH 8.0) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche). The supernatant was incubated with Ni-NTA agarose beads (Qiagen) for 2 hours at 4 ºC. The beads were washed three times with wash buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8.0) and incubated with elution buffer (50 mM sodum phosphate, 150 mM NaCl, 150 mM imidazole) for 15 mins at 4 °C to elute UBA1 proteins.

Tag-Ub-MesNa expression and purification

3×Flag 6×His tagged ubiquitin inserted in pTYB1 plasmid was transformed into Rosetta DE3 cells (EMDmillipore). 1L LB media containing 100 μg/ml ampicillin was inoculated with 50 ml overnight culture and incubated at 37 ºC until OD reached ~1.2. The cell culture media was cooled down to 15 ºC on ice. Then, IPTG (0.1 mM final concentration) was added to the cell culture media, followed by 18 hours incubation at 15 ºC. Cells were then harvested and lysed by sonication in phosphate buffered saline (PBS) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche). The supernatant was incubated with Chitin beads (Pierce Biotechnology) for 1hour at 30 ºC with agitation. The beads were washed three times with washing buffer (HEPES 20mM, NaOAc 50 mM, NaCl 75 mM, pH 6.5) and incubated with 100 mM β-mercaptoethane sulfonic acid (MesNa) overnight at 30 ºC and 150 rpm to cleave the thioester bond and elute the desired Tag-Ub-MesNa. Elution fractions were concentrated (Amicon Ultra spin filter 3K, Millipore), purified by HPLC, and lyophilized providing pure Ub-MesNa.

Synthesis of UBL-Probes

100 μM of UBL-Mes (LC3 or Ub) in 50 mM phosphate buffer (50 mM NaCl, pH 6.5) was treated with **Pro1-3** (final conc. 1 mM) for 2 hours at r.t. Completion of the reaction was monitored by MS analysis of the crude mixture using LC-MS (Bruker AmaZon X Ion Trap). The reaction mixture was dialyzed overnight in 50 mM phosphate buffer (pH 8.0). After the dialysis, LC-MS showed the formation of the disulfide product between two molecules of UBL-Probe-SH. Therefore, TCEP (final conc. 200 μM) was added to reduce the dimeric protein probes. The reaction mixture was then treated with 2,5-dibromohexanediamide (final conc. 1 M) at r.t. for 30 min, following by shaking at 37 °C for 2 hours. When LC-MS analysis showed the complete conversion of the UBL-

Probe-SH, the mixture was dialyzed over night using pH 8.0 phosphate buffer and directly used for the subsequent covalent labeling assays.

Bioassays

Labeling of UBA1 using Ub-Probes

Ub-Probes (final conc. 12 μM) in buffer containing 25 mM HEPES (100 μM TCEP, pH 8.0) was incubated with the wild type UBA1 or C632A UBA1 (1 μM) for 4 hours (10 μl total volume). The resulting reaction mixture was treated with 2 μL of 6× Laemmli loading buffer, and resolved by 12% SDS-PAGE. The proteins in the gel were then visualized by staining the gel with Instant BlueTM.

Labeling test of LC3-Probes with ATG7

LC3-Probes (final conc. 12 μM) in buffer containing 25 mM HEPES (100 μM TCEP, pH 8.0) was incubated with ATG7 or wild type UBA1 (1 μM) for 12 hours (10 μl total volume). The resulting reaction mixture was treated with 2 μL of 6× Laemmli loading buffer, and resolved by 13.5% SDS-PAGE. The proteins in the gel were then visualized by staining the gel with Instant BlueTM.

Click-chemistry protocol to conjugate Rhodamine to UBA-Ub-Probe adduct, following two-color in-gel fluorescent scanning.

Ub-Probes (final conc. 12 μM) in buffer containing 25 mM HEPES (100 μM TCEP, pH 8.0) was incubated with wild type UBA1 or C632A UBA1 (1 μM) for 4 hours (10 μl total volume). The reaction mixture (5 μL) was treated with 5 μL of ubiquitination solution

(UbcH5a 1 μM, Rsp5 0.5 μM, GFP-Sic60 0.2 μM, Ub 25 μM, and ATP 50 μM), and incubated for 15 min. The resulting mixture was treated with 1 μL of 10% SDS, followed by 4 μL of click reaction mixture ($CuSO_4$ (final concentration 1 mM), TBTA (final concentration 100 μM), Sodium Ascorbate (final concentration 1 mM), and Azide-Fluor-585 (final concentration 50 μM)). The resulting reaction mixture was incubated for 30 min, treated with 3 μL of 6× Laemmli loading buffer, and resolved by 12% SDS-PAGE. The bottom of the gel was cut to eliminate the excessive amount of Azide-Fluor-585. The gel was kept in ddH_2O until it was subjected to the in-gel scanning fluorescence imaging (Typhoon 9600, GE Healthcare). The proteins in the gel were then visualized by staining the gel with Instant BlueTM.

(1) An, H.; Statsyuk, A. V. *Chemical Science* **2015**, *6*, 5235.