Supplementary Information

Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes

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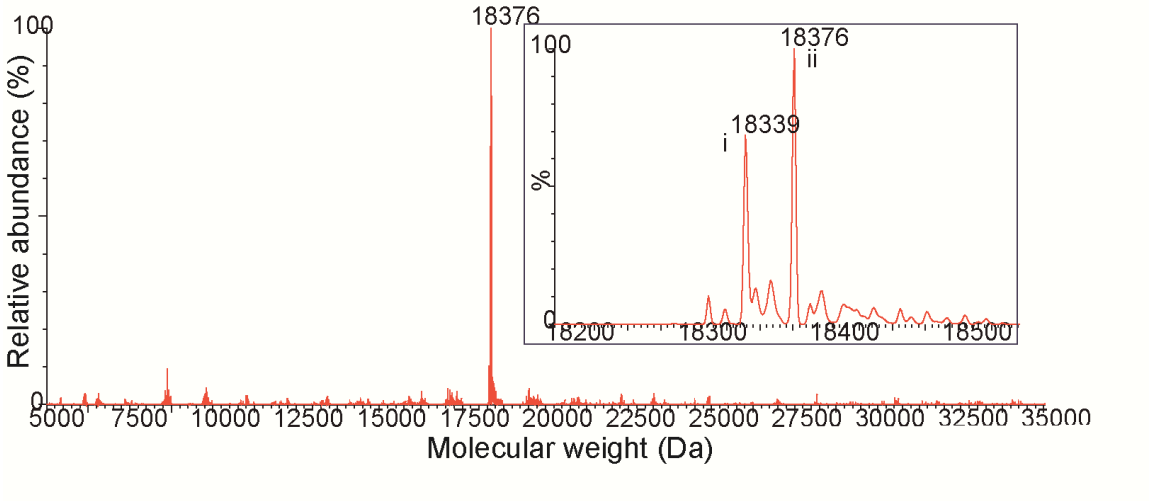


Fig. S1 ESI mass spectrum of HA-tagged K63C-diUb probe. A zoomed-in spectrum is shown in the inset. "i" is the Na^+ adduct (observed = 18,339 Da, calculated = 18,339 Da);"ii" is the Na^+, K^+ adduct (observed = 18,376 Da, calculated = 18,379 Da).

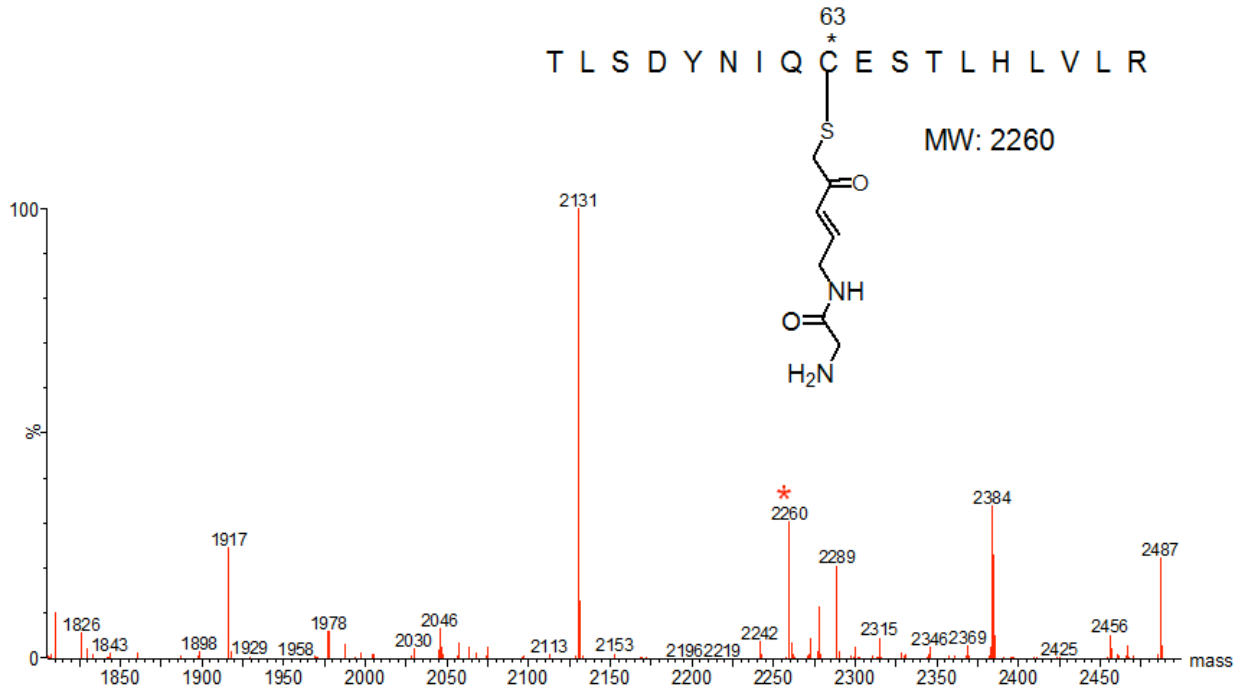


Fig. S2 Deconvoluted electrospray ionization mass spectra of the tryptic peptides of the HA-tagged K63C-diUb probe. The peptide of 2260 Da corresponds to the tryptic fragment that contains the diubiquitin linkage in the HA-tagged K63C-diUb. The mass data were acquired according to the reported methods.[1]

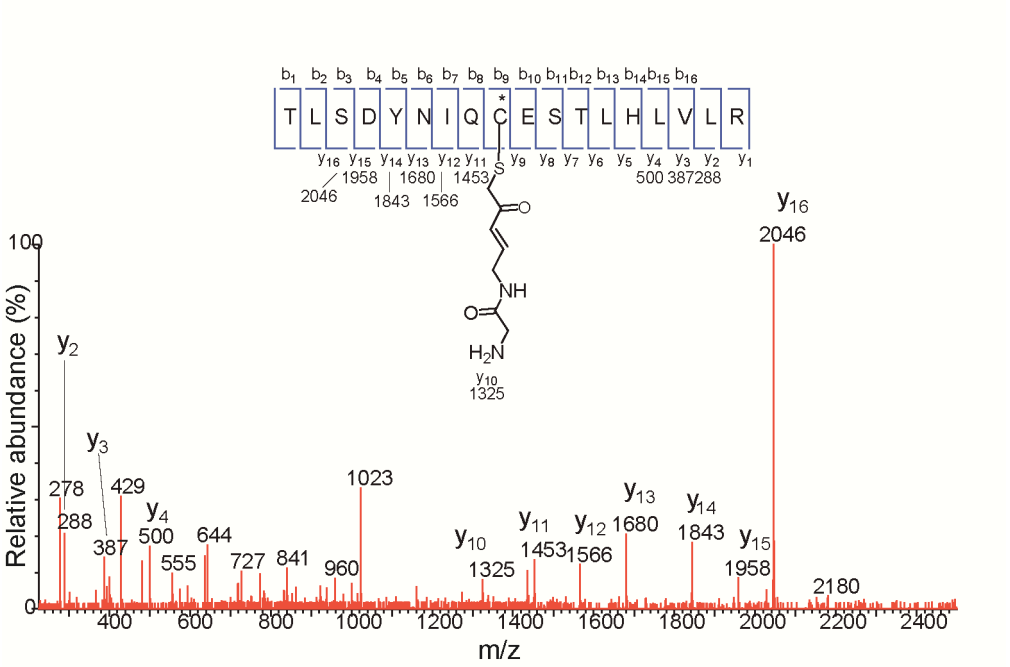


Fig. S3 MS/MS spectra of the tryptic fragment that contains the diubiquitin linkage in the HA-tagged K63C-diUb probe.

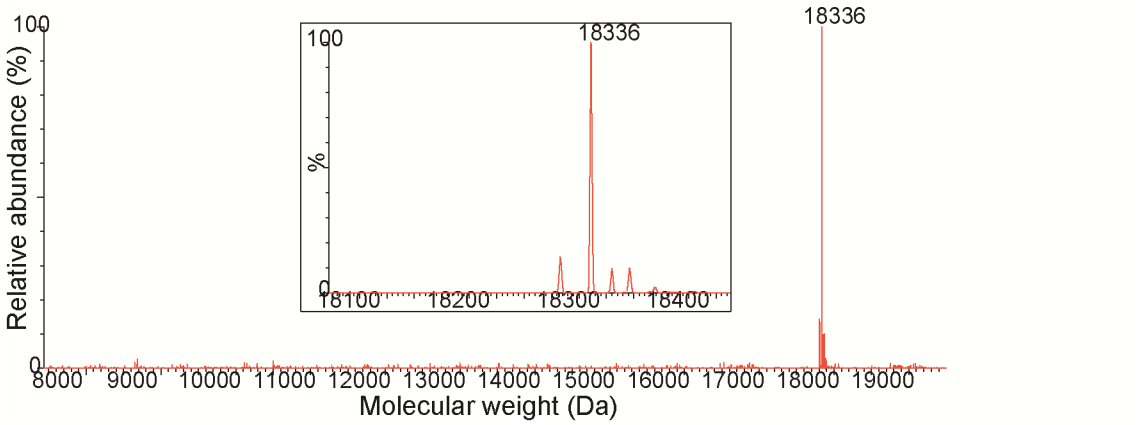


Fig. S4 ESI mass spectrum of HA-tagged K48C-diUb probe. A zoomed-in spectrum is shown in inset. Na^+ adduct (observed = 18,336 Da, calculated = 18,339 Da).

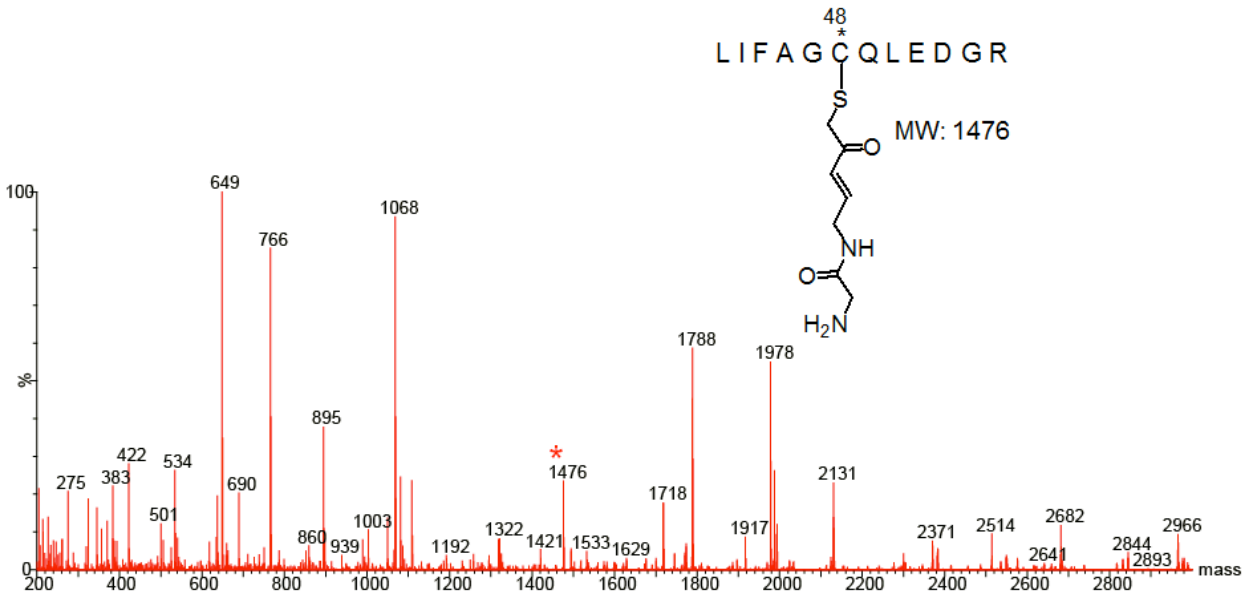


Fig S5. Deconvoluted electrospray ionization mass spectra of the tryptic peptides of the HA-tagged K48C-diUb probe. The peptide of 1476 Da corresponds to the tryptic fragment that contains the diubiquitin linkage in the HA-tagged K48C-diUb.

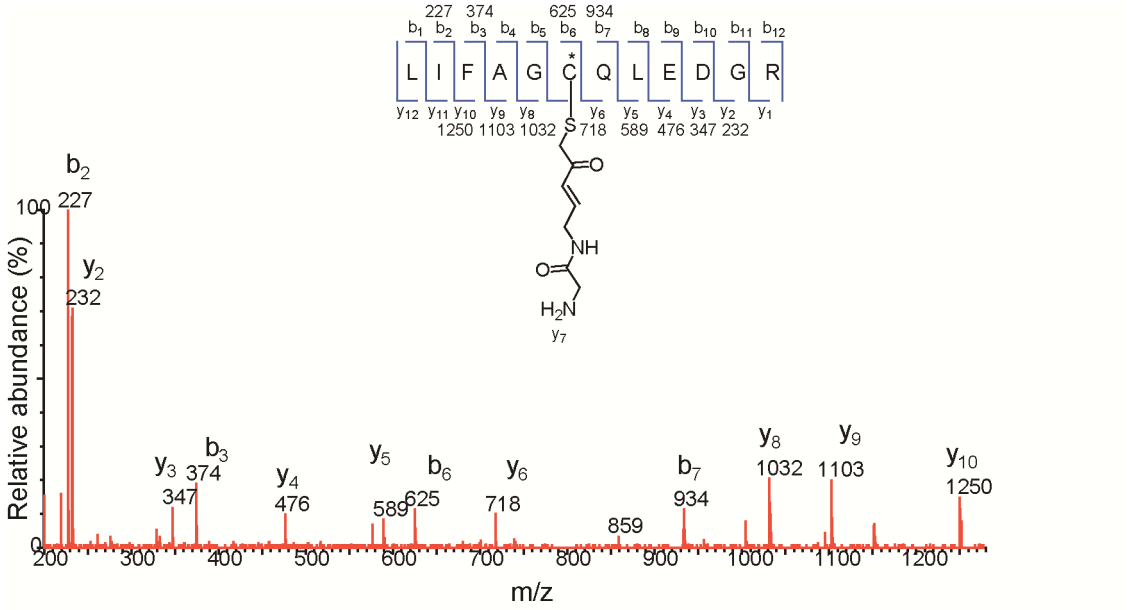


Fig. S6 MS/MS spectra of the tryptic fragment that contains the diubiquitin linkage in the HA-tagged K48C-diUb probe.

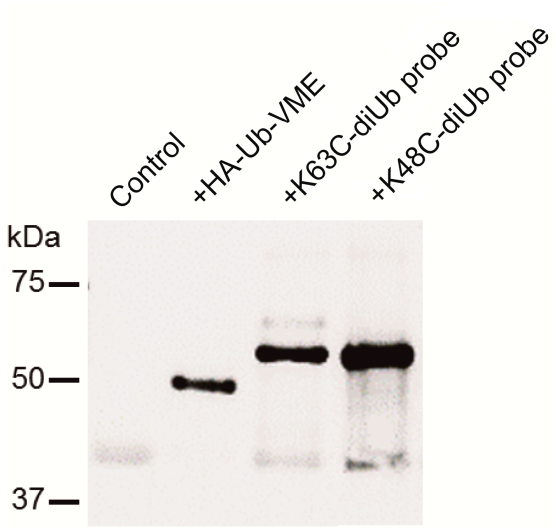


Fig. S7 Western blotting analysis confirming the reactivity of the HA-tagged K63C- and K48C-diUb probes towards the USP2-catalytic core (USP2-CD). USP2-CD was incubated with the probes for 2 hours, separated by SDS-PAGE, and immunoblotted using anti-HA antibody.

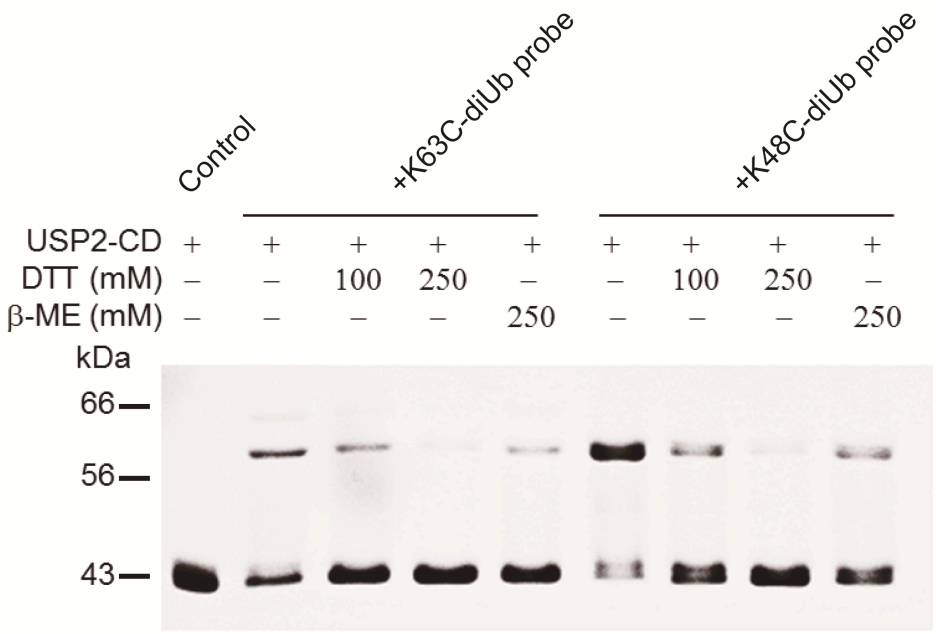


Fig. S8 The thiol-containing agents DTT or β-ME abolished the labeling of USP2-CD by the HA-tagged diUb probes. Different concentrations of DTT (100, 250 mM) or β-ME (250 mM) were incubated with DUBs before the addition of probes.

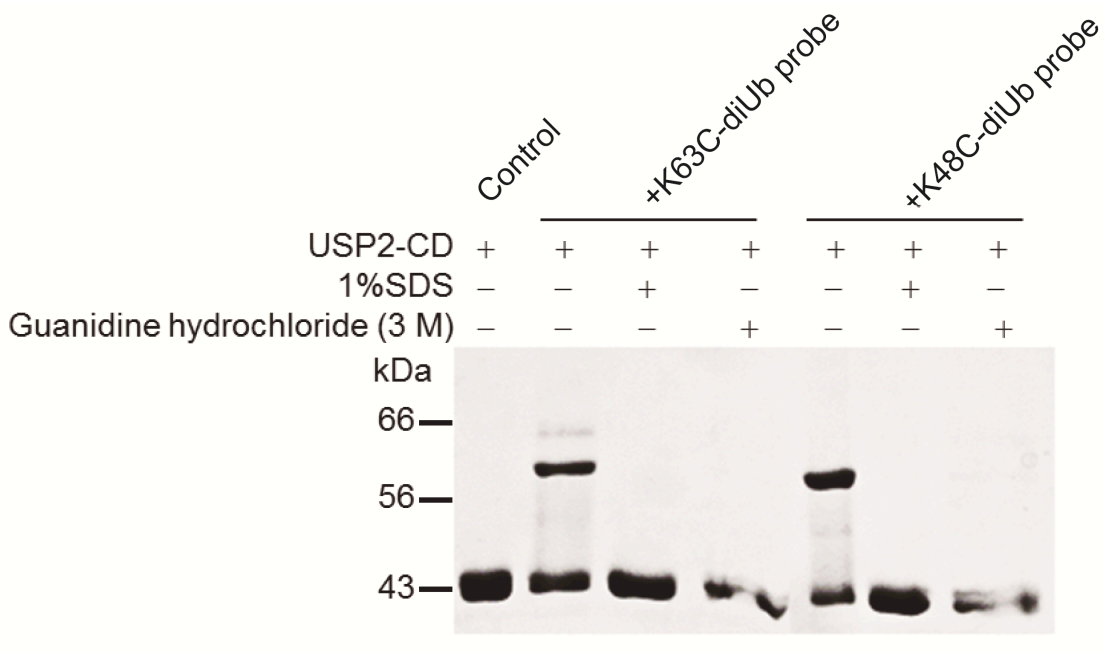


Fig. S9 Effect of denaturing conditions on the reactivity of the HA-tagged diUb probes with USP2-CD. SDS (1%) or guanidine hydrochloride (3 M) was incubated with DUBs before the addition of the diUb probes. The addition of SDS or guanidine hydrochloride abolished the reactivity of diUb probes with USP2-CD, indicating the reactivity was highly dependent on the tertiary fold of the DUBs.

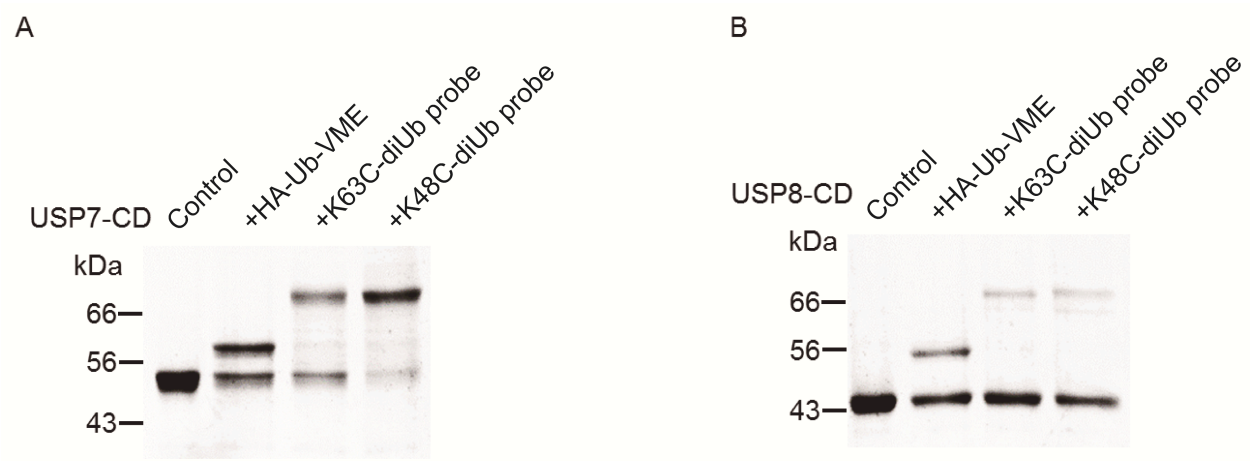


Fig. S10 Labeling of the USP7 catalytic core (USP7-CD) and the USP8 catalytic core (USP8-CD) by the HA-tagged K63C- and K48C-diUb probes. USP7-CD and USP8-CD were incubated with the two diUb probes for 2 hours, separated by SDS-PAGE, and stained with Coomassie blue for detection.

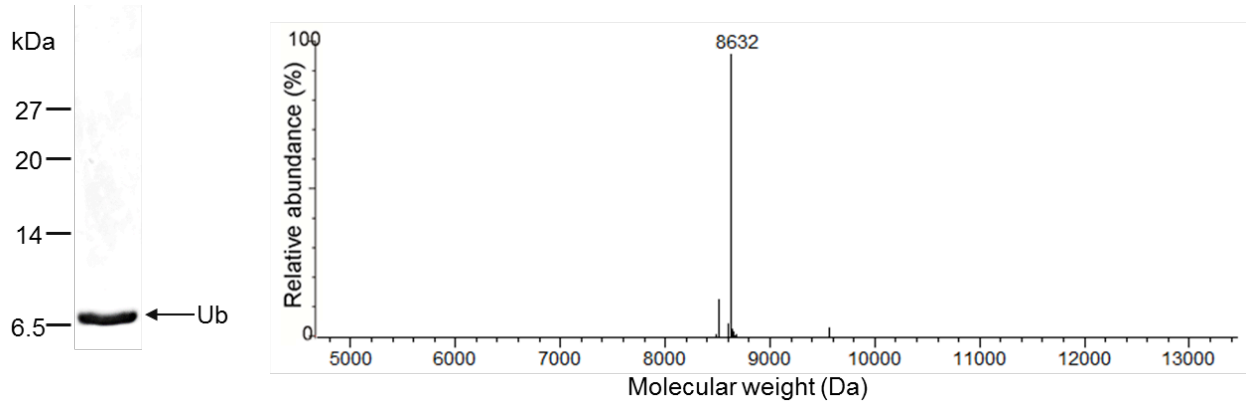


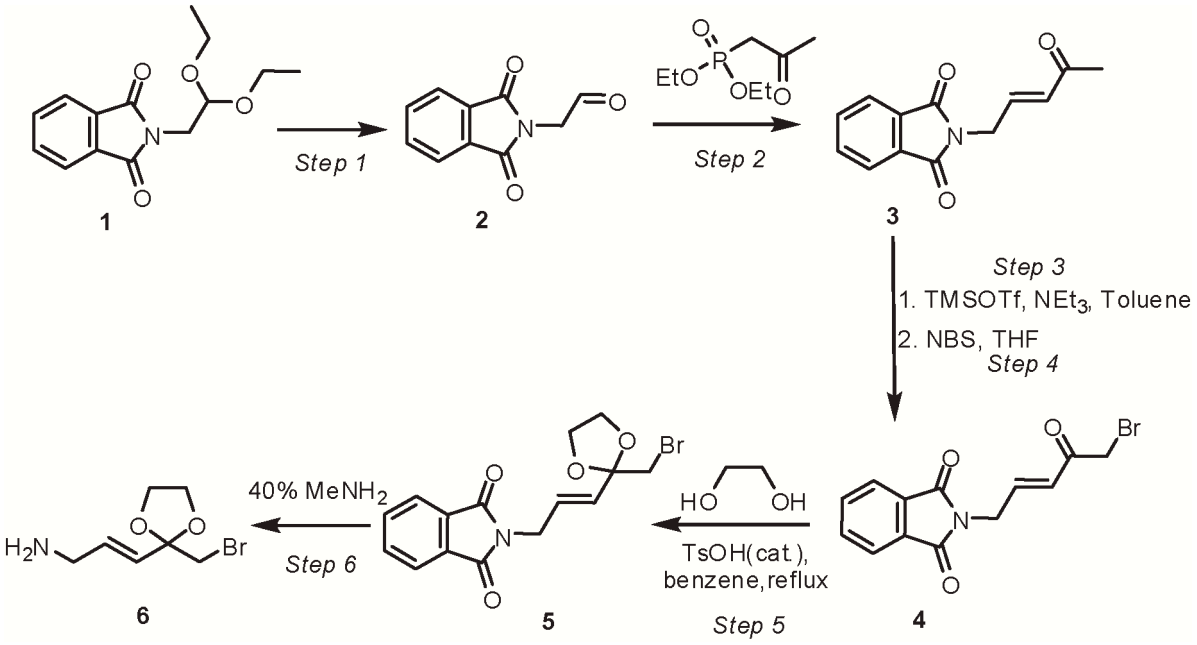
Fig. S11 Gel result and ESI mass spectrum of $hUb_{1\text{-}75}$–MESNA (observed = 8632 Da, calculated = 8632 Da).

Materials and Methods:

1. General Information.

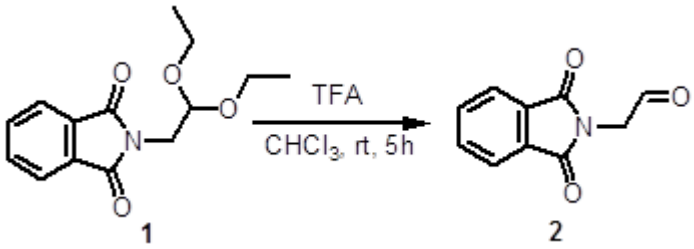
Chemical reagents were obtained from Sigma-Aldrich, Alfa and Acros of the highest available grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV400 NMR Spectrometer with a CryoProbe. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from deuterated solvents as references. Mass spectra were recorded on Shimadzu LCMS 2020 or Waters QTof MS instrument equipped with an electrospray ionization (ESI) source. Analytical thin layer chromatography (TLC) was performed on silica gel 60 GF254 (Merck). Column chromatography was conducted on silica gel (230-400 mesh). Most commercially supplied chemicals were used without further purification. UCH-L1 and OTUB1 were purchased from Boston Biochem.

2. Synthesis of Compound 6



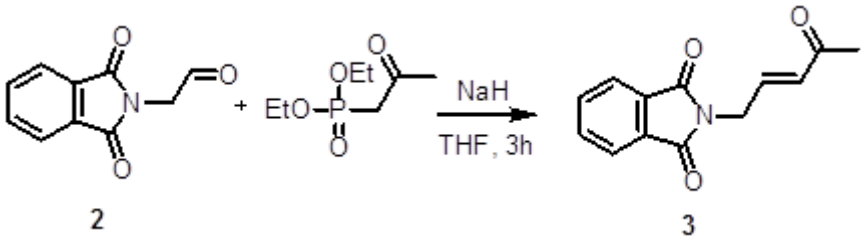
Scheme S1. The route for the synthesis of linker molecule **6**.

2.1. 2-(1,3-dioxoisoindolin-2-yl)acetaldehyde (Compound 2)



Compound **2** was prepared according to a modified method of Veale et al.[2] In an ice/water bath, under a nitrogen atmosphere, to a solution of phthalimido-acetaldehydediethylacetal (13 g, 49 mmol) in $CHCl_3$ (150 mL), TFA (100 mL) was added. The resulting solution was stirred for 1 h. Then the ice bath was removed and the reaction mixture was stirred at r.t. for a further 5 h. The solvent was removed *in vacuo* and co-evaporated with CH_2Cl_2 several times to remove the remaining traces of TFA. This yielded the product as an off-white solid (9 g, 93%). No purification was necessary. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.68 (s, 1H, CHO), 7.91 (m, 2H), 7.78 (m, 2H), 4.59 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ (100 MHz, $CDCl_3$) 193.6, 167.6, 134.4, 131.9, 123.7, 47.4.

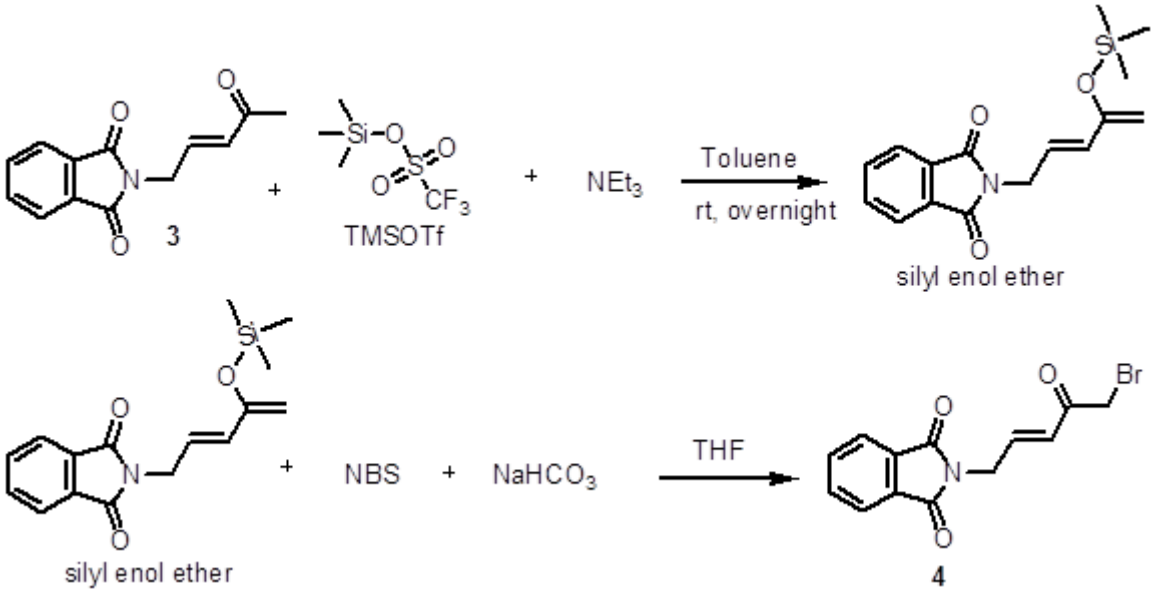
2.2. 2-(4-oxopent-2-enyl)isoindoline-1,3-dione (Compound 3)



Under a nitrogen atmosphere, to a mixture of NaH (560 mg, 22 mmol) and anhydrous THF (90 mL), a solution of diethyl(2-oxopropyl)phosphonate (4.27 g, 22 mmol) in THF (60 mL) was added over 10 min. The resulting solution was stirred at r.t. for 1 h. Then compound **2** (6.24 g, 33 mmol) was dissolved in THF (30 mL) and was added dropwise to the above solution. The reaction mixture was stirred at r.t. for a further 3 h. The reaction was quenched with H_2O, and THF was removed *in vacuo*. The residue was then extracted with CH_2Cl_2 and the combined

organic layer was washed with 1 N HCl and saturated $NaHCO_3$, dried over Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (1:1) to provide compound **3** (4.6 g, 20 mmol, 60 %) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.91 (m, 2H), 7.79 (m, 2H), 6.81-6.74 (m, 1H), 6.16-6.11(dt, *J*=1.6, 16.0 Hz, 1H), 4.49 (dd, *J*=1.6, 4.8 Hz, 2H), 2.27 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 197.7, 167.7, 139.7, 134.4, 131.9, 131.8, 123.7, 123.6, 38.3, 27.2; MS (ESI, positive) *m/z* calcd. for C13H12NO3 $[M+H]^+$: 230, found: 230.

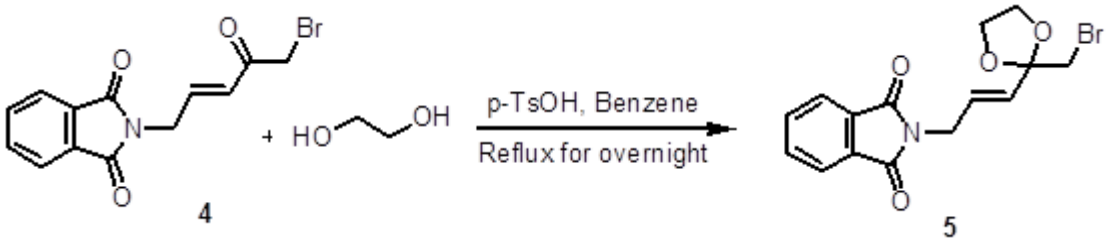
2.3. 2-(5-bromo-4-oxopent-2-enyl)isoindoline-1,3-dione (Compound 4)



Compound **4** was prepared according to a modified method of Little et al.[3] Under N_2 atmosphere, to 2.18 g (9.56 mmol) of compound **3** was added 45 mL of toluene containing 2 mL (14.3 mmol) of NEt_3. Trimethylsilyl triflate 2.25 mL (12.4 mmol) was dissolved in toluene (10 mL) and was added dropwise to the above solution. The reaction mixture was stirred at r.t. for 16 h, and quenched with 100 mL of saturated aqueous $NaHCO_3$. This mixture was extracted with ether (150 mL) three times. The combined organic layer was washed with H_2O, dried with Na_2SO_4, and evaporated. The yellow residual oil was directly used for the next step. In an ice/water bath,

the silyl enol ether was dissolved in 100 mL of anhydrous THF, and 1.2 g (14.3 mmol) $NaHCO_3$ was added. To the mixture was added NBS 1.9 g (10.5 mmol) and the reaction mixture was stirred for 4 h. Then the ice/water was removed and the reaction was quenched with 50 mL of saturated aqueous $NaHCO_3$. The mixture was then extracted with ether (150 mL) for three times. The combined organic layer was washed with H_2O, dried with Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc (3:1) to provide compound **4** (1.46 g, 4.74 mmol, 50%) as a white solid. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.92 (m, 2H), 7.79 (m, 2H), 6.98-6.92 (m, 1H), 6.41-6.36 (dt, *J*=1.6, 15.6 Hz, 1H), 4.53 (dd, *J*=1.6, 5.2 Hz, 2H), 4.01 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 190.1, 167.6, 141.7, 134.4, 131.9, 127.3, 123.7, 38.4, 32.7; MS (ESI, positive) *m/z* calcd. for $C13H11BrNO_3$ $[M+H]^+$: 308, 310, found: 308, 310.

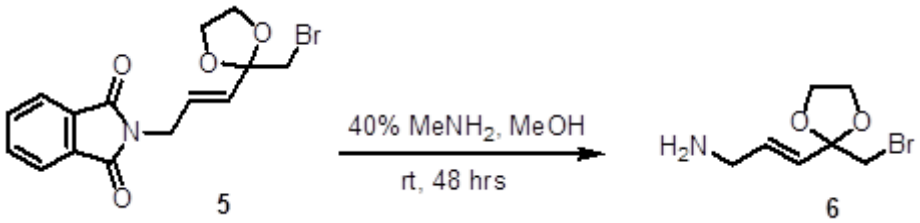
2.4. 2-(3-(2-(bromomethyl)-1,3-dioxolan-2-yl)allyl)isoindoline-1,3-dione (Compound 5)



Under N_2 atmosphere, to the mixture of compound **4** (1.46 g, 4.74 mmol) in 250 mL of benzene was added ethylene glycol (2.94 g, 47 mmol) and p-toluenesulfonic acid (90 mg, 0.47 mmol). The reaction mixture was refluxed for overnight, during which H_2O was removed using Dean-Stark trap. After cooling to room temperature, the reaction was quenched with 50 mL of saturated $NaHCO_3$. The mixture was extracted with ether. The combined organic layer was washed with H_2O, dried with Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography with Hexane/EtOAc(4:1) to provide compound **5** (0.9 g,

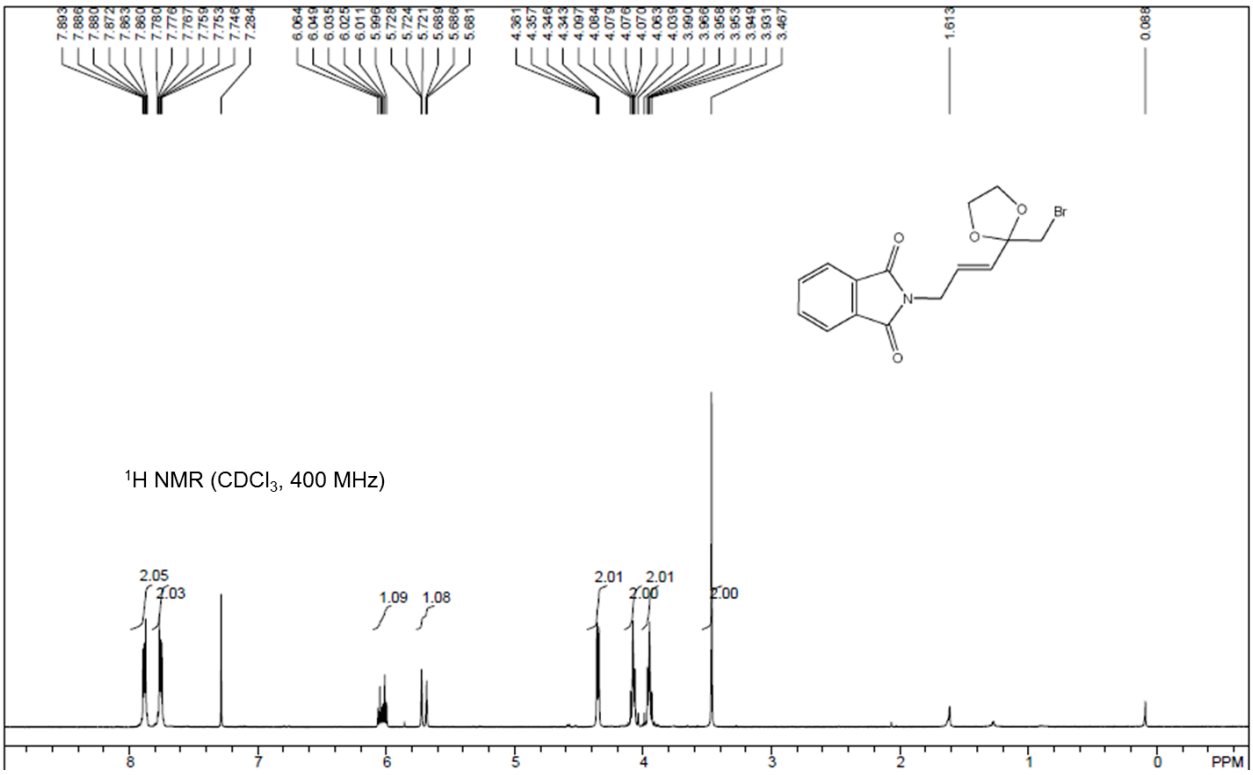
2.56 mmol, 54 %) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.89 (m, 2H), 7.75 (m, 2H), 6.06-6.00 (m, 1H), 5.73-5.68 (dt, *J*=1.6, 15.6 Hz, 1H), 4.35 (dd, *J*=1.6, 6.0 Hz, 2H), 4.10-3.93 (m, 4H), 3.47 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.8, 134.1, 132.0, 130.1, 127.3, 123.4, 105.6, 65.8, 38.4, 36.4; MS (ESI, positive) *m/z* calcd. for C15H15BrNO4 $[M+H]^+$: 352, 354, found: 352, 354.

2.5. 3-(2-(bromomethyl)-1,3-dioxolan-2-yl)prop-2-en-1-amine (Compound 6)

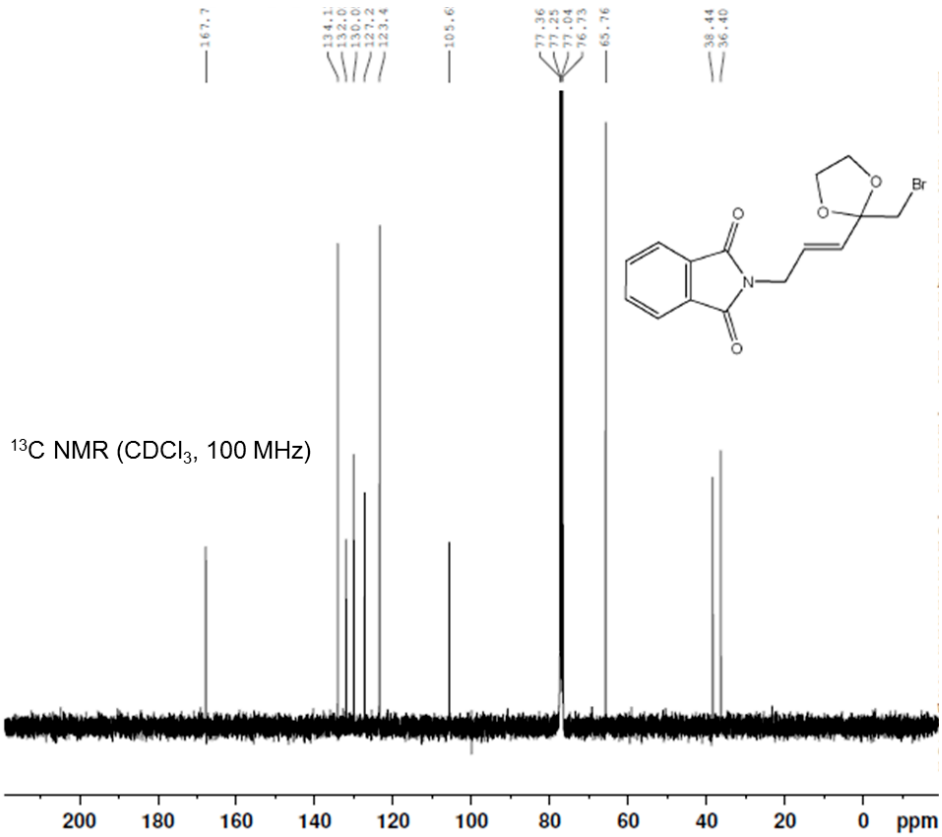


Under N_2 atmosphere, to a mixture of compound **5** (900 mg, 2.56 mmol) and 100 mL of methanol was added 10 mL 40% $MeNH_2$. The reaction mixture was stirred at r.t. for 48 h. The solvent was evaporated and the residue was purified by silica gel column chromatography with NH_3 saturated CH_2Cl_2/CH_3OH (20:1) to provide compound **6** (300 mg, 1.35 mmol, 53 %) as a yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.06-6.00 (m, 1H), 5.57 (d, *J*=15.2 Hz, 1H), 4.04-3.89 (m, 4H), 3.43 (s, 2H), 3.40-3.34 (m, 2H), 1.84 (bs, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 134.6, 133.9, 126.9, 106.0, 105.8, 65.7, 65.4, 42.6, 36.5; MS (ESI, positive) *m/z* calcd. for C7H13BrNO2 $[M+H]^+$: 222, 224, found: 222, 224.

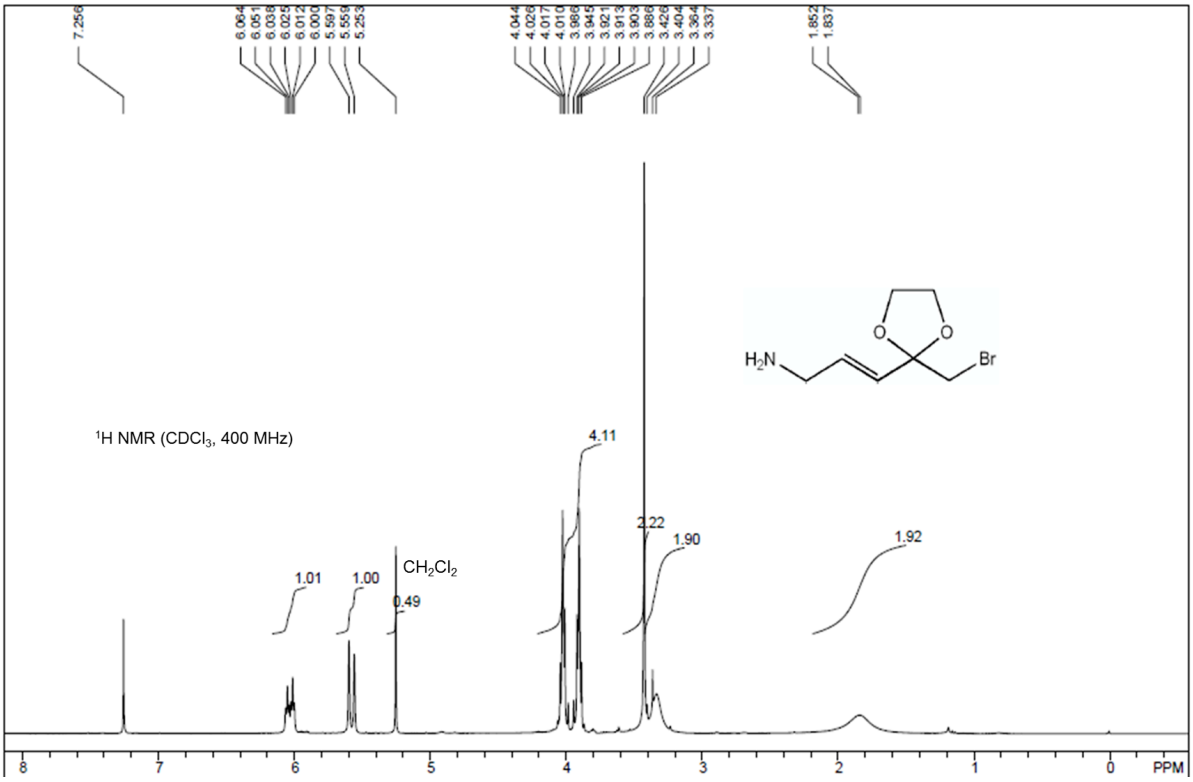
^{1}H NMR of compound 5



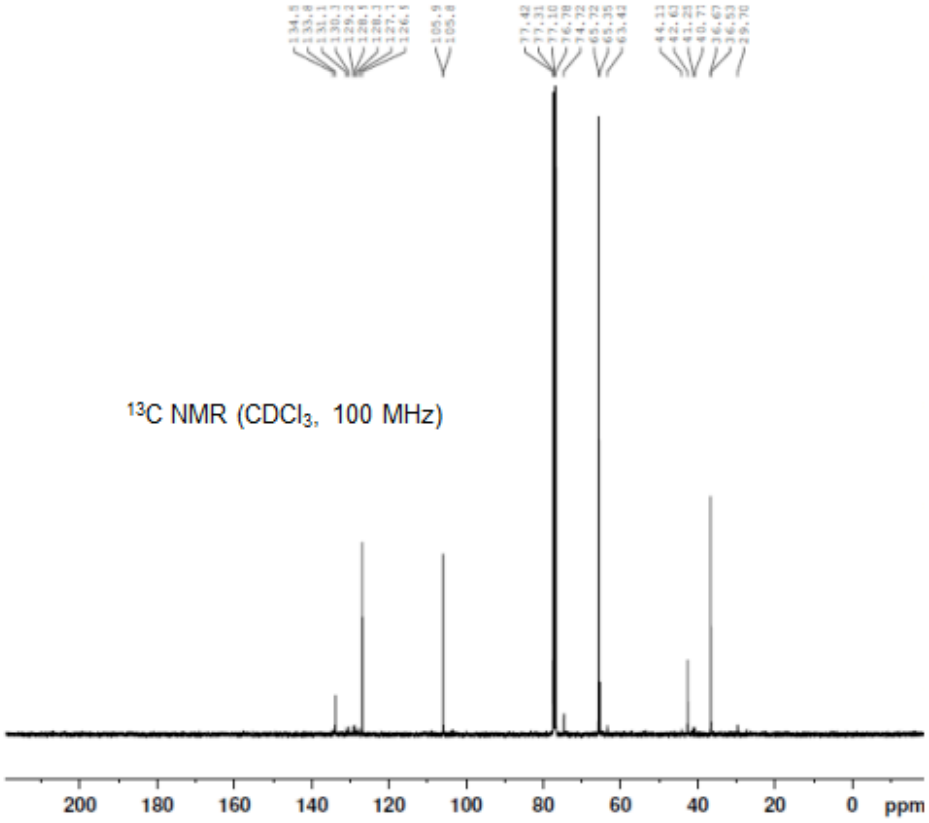
^{13}C NMR of compound 5



^{1}H NMR of compound 6



^{13}C NMR of compound 6



3. Expression and purification of ubiquitin and DUBs

3.1. Generation of hUb_{1-75}–MESNA.

The hUb_{1-75}-pTYB1 plasmid was constructed by cloning the sequence of human Ub (lacking Gly76) into the pTYB1 vector (New England Biolabs). The resulting plasmid was confirmed by DNA sequencing. For protein expression, the plasmid was transformed into BL21(DE3) cells. Cells were cultured at 37 ^{o}C in LB medium (10 L) containing 100 μg/ml ampicillin. The cell culture was induced with 0.4 mM IPTG at OD_{600} of 0.6 ~ 0.8, and grown for an additional 18 hrs at 15 °C. Cells were harvested by centrifugation at 8,000 g for 30 mins and resuspended in lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, 5% glycerol, pH 7.5). Cells were lysed by sonication and the resulting lysate was cleared by centrifugation at 38,000 g for 30 mins. The supernatant was incubated with 50 mL chitin resin (New England Biolabs) at 4 °C for 6 hr. The resin was then washed with 500 ml of high salt wash buffer (20 mM Tris, 1 M NaCl, 1 mM EDTA, 5% glycerol, pH 7.5) and 300 ml of low salt wash buffer (50 mM MES, 100 mM NaCl, pH 6.5). The resin was then incubated with 50 ml cleavage buffer (50 mM MES, 100 mM NaCl, 75 mM β-mercaptoethanesulfonic acid sodium salt (MESNA)) for 12 hrs at room temperature. The molecular weight of the Ub-MESNA species was determined by ESI-MS to 8632 Da (theoretical MW is 8632 Da) (see Figure S11).

3.2. Generation of HA-K63C-hUb_{1-76} and HA-K48C-hUb_{1-76}

HA-K63C-hUb_{1-76} and HA-K48C-hUb_{1-76} plasmids were constructed by Quikchange to generate mutant ubiquitin pTYB1 plasmids (New England Biolabs). The resulting plasmid was confirmed by DNA sequencing. For protein expression, the plasmid was transformed into BL21(DE3) cells. Cells were cultured at 37 ^{o}C in LB medium (10 L) containing 100 μg/ml ampicillin. The cell culture was induced with 0.4 mM IPTG at OD_{600} of 0.6 ~ 0.8, and grown for an additional 18 hrs

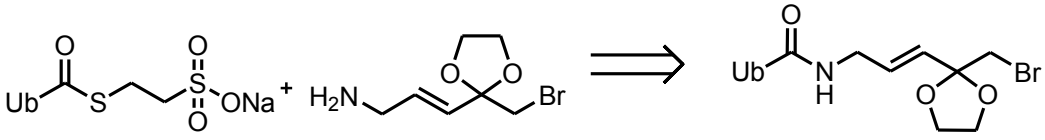
at 15 °C. Cells were harvested by centrifugation at 8,000 g for 30 mins and resuspended in lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, 5% glycerol, pH 7.5). Cells were lysed by sonication and the resulting lysate was cleared by centrifugation at 38,000 g for 30 mins. The supernatant was incubated with 50 mL chitin resin (New England Biolabs) at 4 °C for 6 hr. The resin was then washed with 500 ml of high salt wash buffer (20 mM Tris, 1 M NaCl, 1 mM EDTA, 5% glycerol, pH 7.5) and 300 ml of low salt wash buffer (50 mM Tris, 100 mM NaCl, pH 8.5). The resin was then incubated with 50 ml cleavage buffer (50 mM Tris, 100 mM NaCl, 250 mM DTT, pH 7.7) for 12 hrs at room temperature. The molecular weight of the K63C-hUb$_{1-76}$ species was determined by ESI-MS to 9753 Da (theoretical MW is 9752 Da). The molecular weight of the K48C-hUb$_{1-76}$ species was determined by ESI-MS to 9753 Da (theoretical MW is 9752 Da).

3.3. Expression and purification of USPs

The USP2 and USP7 genes were purchased from Addgene. USP2 (residues 259-605) and USP7 (residues 208-564) were cloned into pET28a following the reported protocols.[4-6] USP8 (residues 734-1110) was kindly provided by Dr. Sirano Dhe-Phaganon.[7] USP21 (residues 209-562) in pET28a-LIC was purchased from Addgene.

4. Synthesis of diUb probes

4.1. Generation of Ubiquitin species 7



MW for Ub-MESNA: 8632

MW: 221

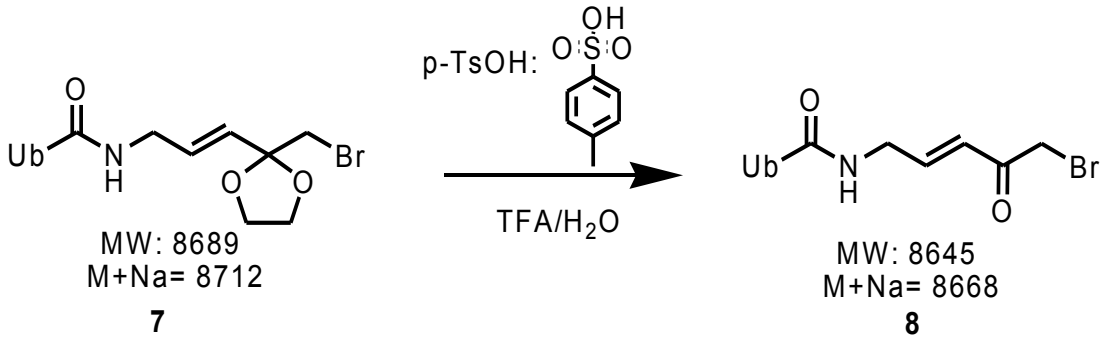
6

MW: 8689

M+Na$^+$= 8712

Before the ligation of hUb$_{75}$-MESNa with compound **6**, hUb$_{1-75}$–MESNa was buffer exchanged with HEPES buffer (pH 6.7) to remove MESNa. To a solution of hUb$_{75}$-MESNa (3 mg/mL) was added 0.4 M of compound **6** (dissolved in the HEPES buffer). The mixture was immediately vortexed and reacted at r.t. overnight. Then the resulting product was buffer exchanged to remove unreacted compound **6**. The molecular weight of the ubiquitin species **7** was determined by ESI-MS to 8713 Da (theoretical MW is 8712 Da).

4.2. Generation of Ubiquitin species 8



Ubiquitin species **7** was added to a solution of p-TsOH dissolved in H_2O and TFA to obtain a solution containing 0.04 M p-TsOH, 54% (v/v) TFA and 0.5 mg/mL ubiquitin species **7**. The reaction was allowed at r.t. for 0.5 hr. The crude mixture was precipitated with ten times volume of cold ether, washed with cold ether and air dried. Then the crude product was dissolved in buffer (100 mM Na_2HPO_4, 8 M urea; 500 mM NaCl, pH 6.0) at a protein concentration of approximately 0.5 mg/ml and dialyzed against folding buffer (20 mM Na_2HPO_4, 100 mM NaCl, pH 6.0). The molecular weight of the ubiquitin species **8** was determined by ESI-MS to 8669 Da (theoretical MW is 8668 Da).

4.3. Generation of the HA-tagged diUb probes

In order to form HA-tagged K63C-diUb probe, ubiquitin species **8** was mixed with K63C-HA-Ub at a concentration of 0.5 mg/mL for both ubiquitin species. The mixture was incubated at r.t. overnight. SDS-PAGE gel was used to detect the formation of diUb. Then the reaction product was purified by a cation-exchange SP Sepharose HP column (GE Life Sciences) using a FPLC system. The protein solution was first buffer exchanged into buffer A (100 mM ammonium acetate, 100 mM NaCl, pH 4.5) using a Centricon (3 kDa MWCO). The sample was loaded to a preequilibrated SP column and then eluted at a flow rate of 1 ml/min using a gradient of 0 to 65% buffer B (100 mM ammonium acetate, 1 M NaCl, pH 4.5). Fractions were collected in 1 ml volume, and those containing pure diubiquitin were pooled and concentrated. HA-tagged K48C-diUb probe was prepared and purified by the same method as described for HA-tagged K63C-diUb probe.

5. DUB labeling assays

5.1. Reactivity of HA-tagged diUb probes towards different DUBs.

DUBs (1 μg) were incubated with 1 μg different probes at room temperature for 2 hours in a reaction buffer containing 50 mM HEPES (pH 7.0), 100 mM NaCl. Then the reaction was quenched by the addition of the SDS-PAGE 6X loading solution. Samples were resolved by reducing SDS-PAGE gel and then stained with Coomassie brilliant blue for detection. For Western blotting the protein samples were transferred to a PVDF membrane and detected by anti-HA antibody as previously described[8].

To evaluate the effect of reducing agents DTT or β-ME on the reactivity of diUb probes with DUBs, 100, 250 mM DTT or 250 mM β-ME was added to DUBs before the addition of probes.

To assess the effect of denaturing conditions on the reaction of diUb probes with DUBs, 1% SDS or 3 M guanidine hydrochloride was added to DUBs before the addition of probes.

5.2. Preparation of HEK293T Cell Extracts and DUB activity-based profiling

This assay was done according to a reported protocol.[9] HEK293T cells were seeded in 10 cm plates, incubated at 37 °C for overnight, and then harvested. Cells were incubated on ice for 1 hour, and lysed on ice in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 2 mM ATP, 0.5% NP40, and 10% glycerol. 50 μg Cell lysates were then incubated with 0.2 μM HA-hUb-VME, HA-tagged K63C-diUb probe and HA-tagged K48C-diUb probe respectively at room temperature for 3 hours in a labeling solution containing 50 mM Tris (pH 7.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 2 mM ATP, and 250 mM sucrose. Samples were separated by SDS-PAGE, transferred onto nitrocellulose membrane and blotted with anti-HA antibody (Sigma). HRP-conjugated anti-mouse (Sigma) was used as the secondary antibody. Signals were detected using ECL Western blotting substrate (Thermo Fisher).

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