Supplemental Information For

Selenocysteine as a Latent Bioorthogonal Electrophilic Probe for Deubiquitylating Enzymes

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General Methods

Rink-amide resin (0.46 mmol/g substitution) was purchased from Chem-Impex (Wood Dale, IL). Fmoc-L-amino acids were purchased from either AnaSpec (Fremont, CA) or AGTC Bioproducts (Wilmington, MA). Other chemical reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich Chemical Company (St. Louis, MO). DNA synthesis was performed by Integrated DNA Technologies (Coralville, IA). Gene sequencing was performed by Genewiz (South Plainfeld, NJ). Plasmid mini-prep, PCR purification and gel extraction kits were purchased from Qiagen (Valencia, CA). Restriction enzymes were purchased from either Fermentas (Thermo Fisher Scientific, Philadelphia, PA) or New England BioLabs (Ipswitch, MA). Reversed-phase HPLC (RP-HPLC) was performed on a Varian (Palo Alto, CA) ProStar HPLC with either a Grace-Vydac (Deerfield, IL) analytical C18 column (5 micron, 150 x 4.6 mm) at a flow rate of 1 ml/min, or a Grace-Vydac preparative C18 column (10 micron, 250 x 22 mm) at a flow rate of 9 ml/min; RP-HPLC was performed using 0.1% trifluoroacetic acid (TFA) in water (A) and 90% acetonitrile, 0.1% TFA in water (B) as the mobile phases. Solid phase peptide synthesis was performed on a Liberty Blue Automated Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC). Mass spectrometric analysis was conducted on a Bruker (Billerica, MA) Esquire, or a Thermo Scientific (Waltham, MA) LTQ Obitrap ESI-MS. Analytical RP-HPLC-mass spectrometry (LC-ESI-MS) was performed on a Hewlett-Packard (Palo Alto, CA) 1100-series LC linked to the Bruker Esquire ESI-MS with an Agilent (Santa Clara, CA) Zorbax C18 column (3.5 micron, 100 x 2.1 mm) using 5% acetonitrile, 1% acetic acid in water (C) and acetonitrile, 1% acetic acid (D) as the mobile phases. NMR spectra were recorded on Bruker Avance AV-300, AV-301, or AV-500 instruments.

Synthesis of L-selenocystine-N-methylamide 1

i. $N^{\alpha},N^{\alpha'}$-di-Boc-L-selenocystine

To a suspension of L-selenocystine (250 mg, 0.748 mmol) in water were added triethylamine (313 µL, 2.244 mmol) and di-tert-butyldicarbonate (500 mg, 2.244 mmol). The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed with 10% HCl. The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (10%(v/v) MeOH in CH_2Cl_2) to obtain compound $N^{\alpha},N^{\alpha'}$-di-Boc-L-selenocystine (361 mg, 90%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.22-6.92 (br, 2H), 5.46-5.73 (br, 2H), 4.56-4.87 (br, 2H), 3.50 (d, J = 10.18 Hz, 4H), 1.50 (s, 18H). ^{13}CNMR (125 MHz, CD_3OD) δ 171.75, 155.10, 78.07, 52.91, 26.21, 26.03. HRMS (ESI):m/z Calcd. for $C_{16}H_{29}N_2O_8Se_2$ 537.0254, found 537.8787 [$M+H^+$].

ii. $N^{\alpha},N^{\alpha'}$-di-Boc-L-selenocystine-N-methylamide

To a solution of N^{α}, $N^{\alpha'}$-di-Boc-L-selenocystine (200 mg, 0.373 mmol) in anhydrous DMF were added methylamine hydrochloride (75 mg, 1.199 mmol), HOBt (176 mg, 1.305 mmol), and triethylamine (182 µL, 1.305 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (203 mg, 1.305 mmol) at 0 °C. The reaction mixture was stirred at

room temperature for 12 h, followed by evaporation of the solvent under reduced pressure. The residue was then dissolved in ethyl acetate and washed with water. The organic solvent was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (1:1(v/v) EtOAc:Hexanes) (142 mg, 68%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (s, 2H), 5.64 (s, 1H), 5.62 (s, 1H), 4.80 - 4.89 (m, 2H), 3.20 (d, J = 6.59 Hz , 4H), 2.84 (s, 3H), 2.83 (s, 3H), 1.48 (s, 18H). ^{13}CNMR (125 MHz, $CDCl_3$) δ 171.08, 156.03, 80.24, 55.64, 36.96, 28.37, 25.92. HRMS (ESI): m/z Calcd. for $C_{18}H_{35}N_4O_6Se_2$ 563.0887, found 563.0874 [M+H$^+$].

iii. L-selenocystine-N-methylamide ***1***

N^{α},$N^{\alpha'}$-di-Boc-L-selenocystine-N-methylamide (100 mg, 0.178 mmol) was stirred in 1:1 (v/v) CH_2Cl_2:TFA at room temperature for 2 hours. Solvent was evaporated under reduced pressure to obtain the trifluoroacetate salt of compound **1** in quantitative yield.

^{1}H NMR (500 MHz, D_2O) δ 4.25 (d, J = 6.27 Hz, 2H), 3.35-3.49 (m, 4H), 2.79 (s, 6H). ^{13}CNMR (125 MHz, D_2O) δ 168.20, 53.50, 28.00, 26.40. HRMS (ESI): m/z Calcd. for $C_8H_{19}N_4O_2Se_2$ 362.9838, found 362.9827 [M+H$^+$].

Synthesis of N^{α}-Boc-L-selenazolidine 6[1]

i. L-selenazolidine

L-selenocystine (1.0 g, 2.99 mmol) was suspended in 38.4 ml of degassed 0.05 M NaOH containing 11.45 mL ethanol. $NaBH_4$ (340 mg, 8.89 mmol) was added slowly over 10 minutes, and the reaction stirred until colorless (20 minutes). The mixture was then cooled in an ice-bath, and excess $NaBH_4$ quenched with the addition of acetic acid. Formaldehyde (90 mmol, 8 mL 37%) was then added, and the reaction allowed for 2 hours under nitrogen atmosphere. Ethanol (150 mL) was then added, and the reaction cooled for several hours in an ice bath. A small amount of red precipitate was filtered off, and then the solvent was removed under reduced pressure. The residue was taken up in 20 mL of ethanol, and cooled to give a cream colored solid, which was vacuum filtered and used without further purification (942 mg, 87%).

ii. N^{α}-Boc-L-selenazolidine ***6***

L-selenazolidine (1 g, 5.56 mmol) was taken up in 15 mL of H_2O. The solution was cooled and diisopropylethylamine (16.48 mmol) was added, followed by di-tert-butyldicarbonate (16.68 mmol) in 2 mL of methanol. The reaction was allowed to proceed under nitrogen atmosphere for 16 hours, after which the solvent was removed under high vacuum. The residue was taken up in ethyl acetate (100 mL), washed with 1M HCl (18 mL), H_2O (20 mL), then dried and evaporated *in vacuo*. The crude material was purified by column chromatography (1%(v/v) MeOH in DCM) to obtain **3** (654 mg, 39%. Combined 34% over steps i and ii).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.85 (bs, 1H, -COOH), δ 5.14-5.36 (m, 1 H, C(O)CαHN), δ 4.88 (bd, 1H, $SeCH_2N$), δ 4.50 (bd, J = 7.35, 1H, $SeCH_2N$), δ 3.38-3.40 (dd, J = 3.42, $SeCH_2C\alpha$), δ 3.32 (m, 1 H, $SeCH_2C\alpha$), 1.48 (s, 9 H, O^{t}Bu). ^{13}CNMR (500 MHz, $CDCl_3$) δ 176.02, 175.49 (COOH), 153.94, 153.59 (NC(O)O), 81.85 (O*C*$(CH_3)_3$), 62.74 (Cα), 39.92, 38.69 ($SeCH_2N$),

28.25 ($OC(CH_3)_3$), 25.19, 24.33 ($SeCH_2C\alpha$). ESI HRMS calcd. m/z for $C_9H_{16}NO_4Se$ 282.0244282 $[M+H]^+$, found 181.9724 $[M\text{-}Boc]^+$, 225.9627 $[M\text{-}tBu]^+$.

Molecular cloning

FLAG-Ub(1-75)-MxeGyrA-CBD plasmid

A FLAG tag (MDYKDDDDKA) was introduced by PCR amplification of the Ub(1-75) sequence from previously reported pTXB1-Ub(1-75)-*Mxe*GyrA-CBD plasmid[2] using the following mutagenic primers:

Primer	DNA Sequence (5'- to -3')
FLAG-Ub(1-75)-FP	CTTTAGAAGGAGATATA*CATATG*GATTACAAGGATGACGACGATAAA GCGATGCAGATCTTCGTGAAGACTCTG
FLAG-Ub(1-75)-RP	GGTGGTT*GCTCTTC*CGCAACCTCTGAGACGGAGTACCAGGTGCAG GGT

The PCR product was digested with *NdeI* and *SapI* restriction endonucleases, treated with Calf Alkaline Intestinal Phosphatase, and then gel purified. This product was ligated into a similarly digested pTXB1 vector with T4 DNA ligase. The ligation mix was used to transform ultracompetent DH5 alpha *E. coli* cells. The correct DNA sequence for FLGA-Ub was verified by T7 forward primer-based DNA sequencing.

Protein overexpression and purification

Overexpression of FLAG-Ub(1-75)-MxeGyrA-CBD and purification of FLAG-Ub(1-75)-MESNa α-thioester

Overexpression was achieved in BL21 *E. coli*, which were grown at 37 ºC to an OD_{600} of 0.4, then cooled to 16 ºC and induced over 9 h with 0.3 mM IPTG. Cells were harvested by centrifugation at 6,000x*g* for 15 min at 4 ºC. The cell pellet was resuspended in 22 mL lysis buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and lysed by sonication, and centrifuged for a further 30 min at 20,000x*g* at 4 ºC. The lysate supernatant was passed through a 0.45 µm filter then applied to a 10 mL chitin column pre-equilibrated with lysis buffer and incubated overnight at 4 ºC. The column was first washed with lysis buffer (20 Column Volumes, CV), then 35 mL thiolysis buffer was applied to the column (100 mM sodium 2-mercaptoethanesulfonate (MESNa), 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The first elution was collected after 48 h at 4 ºC, and a second 35 mL of thiolysis buffer was applied for a further 48 h. The combined eluted protein was purified by C18 Preparative RP-HPLC (30-70% B, 1 h gradient) to yield 9.2 mg FLAG-Ub(1-75)-MESNa α-thioester from 3 L of growth media.

Overexpression of UCH-L3 WT and C95A mutant

Wild-type UCH-L3 and the C95A mutant were expressed as previously described.[2]

Expression and purification of His_6-MBP-USP15

USP15 was overexpressed in BL21(DE3) cells with an N-terminal His_6-MBP (maltose binding protein) tag. Cells were grown at 37ºC with shaking at 180 rpm until OD_{600} reached 0.4. Protein

expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.2mM). Cells were grown overnight at 16 ºC, and then harvested by centrifugation for 30 minutes at 4,000x*g*, 4 ºC. The cell pellet was resuspended in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1mM TCEP, and 0.25 mM PMSF) and lysed by sonication. The lysate was clarified by centrifugation for 30 min at 75,000x*g*, 4 ºC. USP15 was then purified using two chromatographic steps: nickel affinity chromatography followed by anion exchange after the MBP fusion was removed using TEV protease. Enzyme activity was confirmed with the fluorogenic probe Ub-AMC, as previously described.[3]

Expressed protein ligation of 1 and Ub(1-75)-MESNa α-thioester.[4]

Ub(1-75)-MESNa α-thioester (0.5 mM) was reacted with **1** (1 mM) in ligation buffer (6 M Gn-HCl, 200 mM NaPi, 200 mM mercaptophenylacetic acid (MPAA), pH 7.5) at 25 ºC. After 12 h the reaction was brought to 1 M in dithiothreitol (DTT), and adjusted to pH 8 for a further 12 h at 25 ˚C. The reaction was purified by C18 analytical RP-HPLC (30-55% B, 30 min gradient) (76%).

Oxidative conversion of Ub-SeCys-*N*-methylamide to Ub-Dha-*N*-methylamide 4

Ub-SeCys-*N*-methylamide (0.35 mM) was sonicated to give a heterogeneous suspension in 100 mM NaOAc, pH 5.5. To this suspension was added a solution of $NaIO_4$ in 100 mM NaOAc, pH 5.5, such that the final concentration of $NaIO_4$ was 0.35 mM. The suspension was allowed to react for 2 hr at 25 ˚C protected from light, after which time crystalline Gn-HCl was added to a concentration of 6 M. Following denaturation the reaction was allowed to proceed for a further 48 hr to allow Se elimination to occur. The reaction was purified by C18 analytical RP-HPLC (30-55% B, 45 min gradient) to obtain Ub-Dha-*N*-methylamide, **4**, in 37% yield.

Solid-phase peptide synthesis

Synthesis of AcHN-TRIM25(112-124)- Rink-amide resin

The peptide AcHN-KEAAVK(SeCys)TCLVCMA-CONH2 was synthesized by microwave-assisted SPPS on a 0.1 mmol scale utilizing standard 9-fluorenylmethoxycarbonyl (Fmoc)-based N^{α}-deprotection chemistry. Starting from Rink-amide resin (0.22 g, 0.46 mmol/g) each amino acid was coupled in 5.25 molar excess based on resin loading. Deprotection of the Fmoc- group was achieved by treating resin with 20% piperidine in DMF for 65 s at 90 °C. Coupling reactions were undertaken for 2 min at 90 °C with a mixture of Fmoc-amino acid (0.53 mmol), O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU, 0.51 mmol) and N,N-Diisopropylethylamine (DIEA, 1.1 mmol) in DMF. The N^{ε} of K117 was orthogonally protected with the 1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-3-methylbutyl (ivDde) protecting group. The peptide was N-terminally acetylated by incubating the deprotected resin twice, for 1 h each time, with acetic anhydride (2 mmol) and DIEA (2 mmol) in DMF.

Installation of K117-iso-SeCys

Deprotection of N^{ε} of K117 was accomplished by reacting resin bound peptide with a solution of 5% NH_2NH_2 in DMF overnight. **6** (0.15 mmol) was coupled to K117 N^{ε} with *N,N′*-

Diisopropylcarbodiimide (DIC, 0.15 mmol) and Ethyl cyano(hydroxyimino)acetate (Oxyma, 0.15 mmol) overnight. Complete coupling was confirmed by small-scale test cleavage, HPLC purification and subsequent ESI-MS. The remaining resin-bound peptide was cleaved and deprotected with Reagent K consisting of 82.5:5:5:5:2.5(v/v) ratio of TFA: thioanisole: H_2O: phenol: 1,2-ethanedithiol, for 1 h at room temperature. The peptide was precipitated with ice-cold diethyl ether, centrifuged to pellet and washed further with ice-cold diethyl ether. The dried peptide was taken up in 6 M Gn-HCl, 100 mM NaOAc (pH 4.0), 50 mM tris(2-carboxyethyl)phosphine (TCEP), and purified by C18 preparative RP-HPLC (0-73% B, 60 min) to yield pure peptide in 13% overall yield based on the initial resin loading. Deprotection of the acetal moiety was accomplished by dissolving the peptide (2 mM) in 5 M Gn-HCl, 1.6 M Methoxylamine HCl, pH 5.0 and nutating the solution for 4 h at 25 ^{o}C. Immediately prior to purification, samples were chilled on ice and reduced with TCEP (10 mM) buffered at pH 7.0 for 30 s. C18 preparative RP-HPLC (0% B, 15 min; 0-100% B, 30 min) of the freshly reduced material resulted in 75-80% recovery of the TRIM25(112-124) K117-iso-SeCys peptide **7**.

Expressed protein ligation of TRIM25(112-124) K117-iso-SeCys 7 and Ub(1-75)-MESNa α-thioester 2

Peptide **7** (2.5 mM, 1.2 mg) was ligated with Ub(1-75)-MESNa α-thioester (0.5 mM, 2.2 mg) in 500 μL of degassed ligation buffer (6 M Gn-HCl, 100 mM MPAA, 100 mM NaPi, 1 mM EDTA, 5% DMF, pH 7.5) at 25 ^{o}C. After 24 h, the reaction was acidified with phosphoric acid to pH 3.0 and washed five times with diethyl ether to remove excess MPAA. The reaction was then chilled on ice and reduced for 30 s with buffered TCEP (25 mM). The reduced reaction mixture was immediately purified by C18 Analytical RP-HPLC (20-60% B, 30 min gradient) to yield 1.1 mg of the ligation product **8** in 42.6% overall yield.

Synthesis of Ub-Dha-TRIM-25 9

Ub-SeCys-TRIM-25 (0.3 mM, 3 mg/ml) in 8 M Urea, 100 mM NaOAc, pH 3.4 was chilled on ice and reduced with TCEP (15 mM) for 10 min. A solution of α, α-di-bromoadipoyl(bis)amide[5] (280 mM) in DMF was added to 10%(v/v) of the total reaction volume, and the reaction was incubated on ice inside a 4 ^{o}C refrigerator for a further 12 hr. The reaction was purified by C18 analytical RP-HPLC using a gradient of 30-50% B over 30 min to yield the desired probe **9** in 27% overall yield.

General protease labeling

The probe Ub-Dha-*N*-methylamide **4** (1.8 nmol) was solubilized in a volume of 8 M Urea that is 5% of the final reaction volume. Various DUBs (0.252 nmol) were activated with 1 mM buffered TCEP for 10 min at 25 ^{o}C, and then added to the solubilized probe. Reactions were allowed in a 37 ^{o}C water bath for 12 h, and stopped by the addition of TFA to 0.2%(v/v) final concentration. Laemmli sample buffer was added and samples were boiled for 5 minutes with 100 mM TCEP prior to analysis by SDS-PAGE and Western blots.

USP15 labeling

The probe **9** (1 mg) was solubilized in 8 M Urea to prepare a 2 mM stock. It was further diluted in reaction buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) to make 200 µM working stock. USP15 (1.2 µM) was incubated in the reaction buffer for 10 min at 25 ^{o}C, followed by the addition of the probe (70 µM), and the reaction was allowed to proceed for 12 h at 37 ˚C. Laemmli sample buffer was added and samples were boiled for 5 minutes with 10 mM DTT prior to analysis by SDS-PAGE and Western Blot.

UCH-L3 labeling in human cell lysate

HeLa cells were cultured under standard conditions and collected by trypsinization followed by centrifugation. The pellet was washed twice with PBS, then cells were resuspended in lysis buffer: 50 mM tris pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM methionine, 1 mM PMSF, 0.5% IGEPAL CA-630. Cells were lysed by brief sonication on ice, and lysate clarified by centrifugation at 13,500 rpm, 4 °C, for 20 min. The supernatant was collected, and glycerol added to 20% (v/v). Total protein concentration in the lysate was determined by Bradford assay.

Labeling reactions were performed in 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.5. Lysate was added to a concentration of 1 ug/uL. FLAG Ub-Dha was added to the desired concentration from a stock solution of FLAG-Ub-Dha dissolved in 50:50 H_2O: ACN. Reactions were incubated at 37 °C for 16 h, then quenched with SDS loading dye containing DTT (50 mM final concentration) and boiled for 5 min. Samples were loaded on a 12% SDS-PAGE gel and run at 200 V for 45 min. Protein was transferred to PVDF membrane (Bio-Rad #162-0177) at 4 °C for 12 h at 35 V in Towbin blot buffer with SDS, then immunoblotted with rabbit anti-UCH-L3 (1: 1,000 dilution, Cell Signaling Technologies #8141). The membrane was incubated with secondary HRP-conjugated anti-rabbit (1: 40,000 dilution, Jackson Immunoresearch Laboratories #111-035-003). The blot was visualized by enhanced chemiluminescence with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific #34075) and Kodak BioMax Light autoradiography film (Carestream Health #819-4540).

HeLa whole-cell proteome labeling competition assays

*Competition of **5** with N-Ethylmaleimide and 2-Iodoacetamide*

HeLa cells were cultured under standard conditions and collected by trypsinization followed by centrifugation. The pellet was washed twice with PBS, then cells were resuspended at $3x10^6$ cells per 100 µL in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM Methionine, 1 mM PMSF, and 0.5% (v/v) IGEPAL CA-630. A DUB-inhibited lysate was prepared by lysing cells in an identical lysate buffer supplemented with 20 mM 2-Iodoacetamide (IA) and 10 mM N-Ethylmaleimide (NEM). Cells were incubated on ice for 20 min then lysed by brief sonication. The lysate was clarified by centrifugation in a 4 ^{o}C microcentrifuge at 13,500 rpm for 20 min. The total protein concentration in lysates was determined by a Bradford assay.

DUB-labeling assays were performed in 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.5. To 100 µg of clarified lysate, FLAG Ub-Dha (**5**) was added to a final concentration of 100 µM from a stock solution of FLAG-Ub-Dha dissolved in 50:50 (v/v) H_2O:ACN (≤2% total reaction volume). Reactions were incubated at 37 °C for 90 minutes and then quenched with four volumes of 8 M

Gn-HCl. Samples were purified by Analytical RP-HPLC (Grace-Vydac, C4, 5 micron, 150 x 4.6 mm) over a 15 minute 10-100% B gradient in order to reduce signal from unreacted **5**. The entire collected eluate was lyophilized, resolublized in 40 μL of 8 M Urea and 100 mM DTT, and mixed with 8 μL of 6x Laemmli gel-loading dye. The mixture was heated for 10 min at 60 ˚C and samples were resolved by 10% SDS-PAGE, run at 200 V for 40 min. Protein was transferred to PVDF membrane (Bio-Rad #162-0177) at 4 °C for 12 h at 35 V in Towbin blot buffer with SDS, then immunoblotted with rabbit anti-FLAG (1: 4,000 dilution, Sigma #F2555). The membrane was incubated with secondary HRP-conjugated anti-rabbit (1: 40,000 dilution, Jackson Immunoresearch Laboratories #111-035-003). The blot was visualized by enhanced chemiluminescence with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific #34075) and Kodak BioMax Light autoradiography film (Carestream Health #819-4540).

*Competition of **5** with Ub-VME*

HeLa cells were cultured under standard conditions and collected by trypsinization followed by centrifugation. The pellet was washed twice with PBS, then cells were resuspended at $3x10^6$ cells per 100 μL in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM Methionine, 1 mM PMSF, and 0.5% (v/v) IGEPAL CA-630. Cells were incubated on ice for 20 min then lysed by brief sonication. The lysate was clarified by centrifugation in a 4 ^oC microcentrifuge at 13,500 rpm for 20 min. The total protein concentration in lysates was determined by a Bradford assay.

Competition assays were performed in 100 mM Na_2HPO_4, 150 mM NaCl, pH 7.5. A stock solution of buffered Ub-VME (Boston Biochem, U-203) was added to **5** dissolved in 50:50 H_2O: ACN (≤2% total reaction volume) such that the final assay concentrations were 23 μM in Ub-VME and either 23 μM or 100 μM in **5**. To the combination of probes in reaction buffer were added 100 μg total lysate protein. Assays were incubated at 37 °C for 90 min and quenched with four volumes of 8 M Gn-HCl. Samples were purified by Analytical RP-HPLC (Grace-Vydac, C4, 5 micron, 150 x 4.6 mm) over a 15 minute 10-100% B gradient in order to reduce signal from unreacted **5**. The collected eluate was lyophilized and then dissolved in 40 μL of 8 M Urea, 100 mM DTT. The resulting solution was mixed with 8 μL of 6x Laemmli gel-loading dye, and heated for 10 min at 60 ˚C. Samples were resolved by 10% SDS-PAGE run at 200 V for 40 min. Proteins were transferred to PVDF membrane (Bio-Rad #162-0177) at 4 °C for 12 h at 35 V in Towbin blot buffer with SDS, then immunoblotted with rabbit anti-FLAG (1: 4,000 dilution, Abcam #ab1162). The membrane was incubated with secondary HRP-conjugated anti-rabbit (1: 40,000 dilution, Jackson Immunoresearch Laboratories #111-035-003). The blot was visualized by enhanced chemiluminescence with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific #34075) and Kodak BioMax Light autoradiography film (Carestream Health #819-4540).

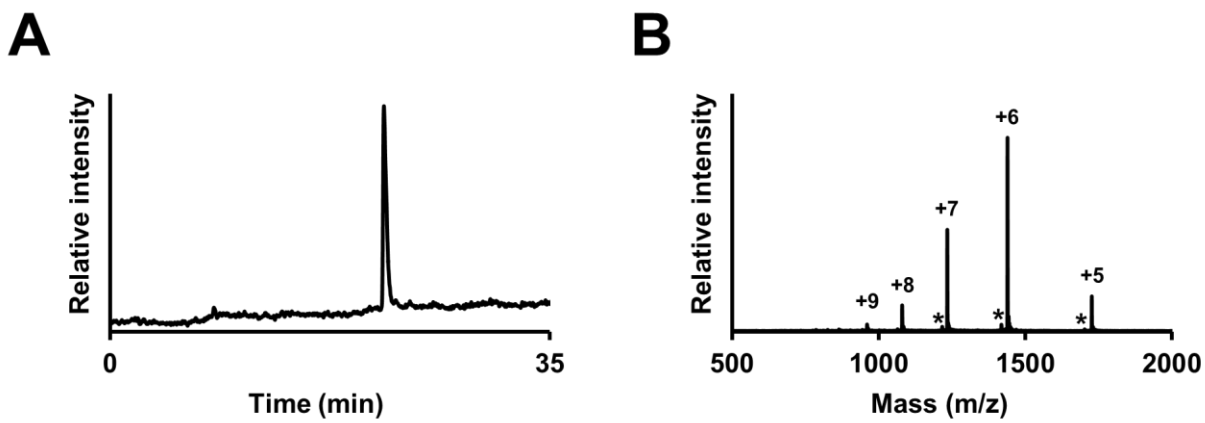


Figure S1. **Purification of Ub(1-75)-MESNa α-thioester 2**. (**A**) C18 Analytical RP-HPLC chromatogram of purified Ub(1-75)-MESNa α-thioester, 30 min. 0-73% B gradient. (b) ESI-MS of purified Ub(1-75)-MESNa α-thioester. Observed [$M+H^+$] 8,633.3 ± 1.8 Da. Calcd. 8,632.0 Da. Asterisks (*) indicate hydrolyzed Ub(1-75)-CO_2H. Observed [$M+H^+$] 8,508.5 ± 1.9 Da. Calcd. 8,507.7 Da. All reported masses are isotopic averages.

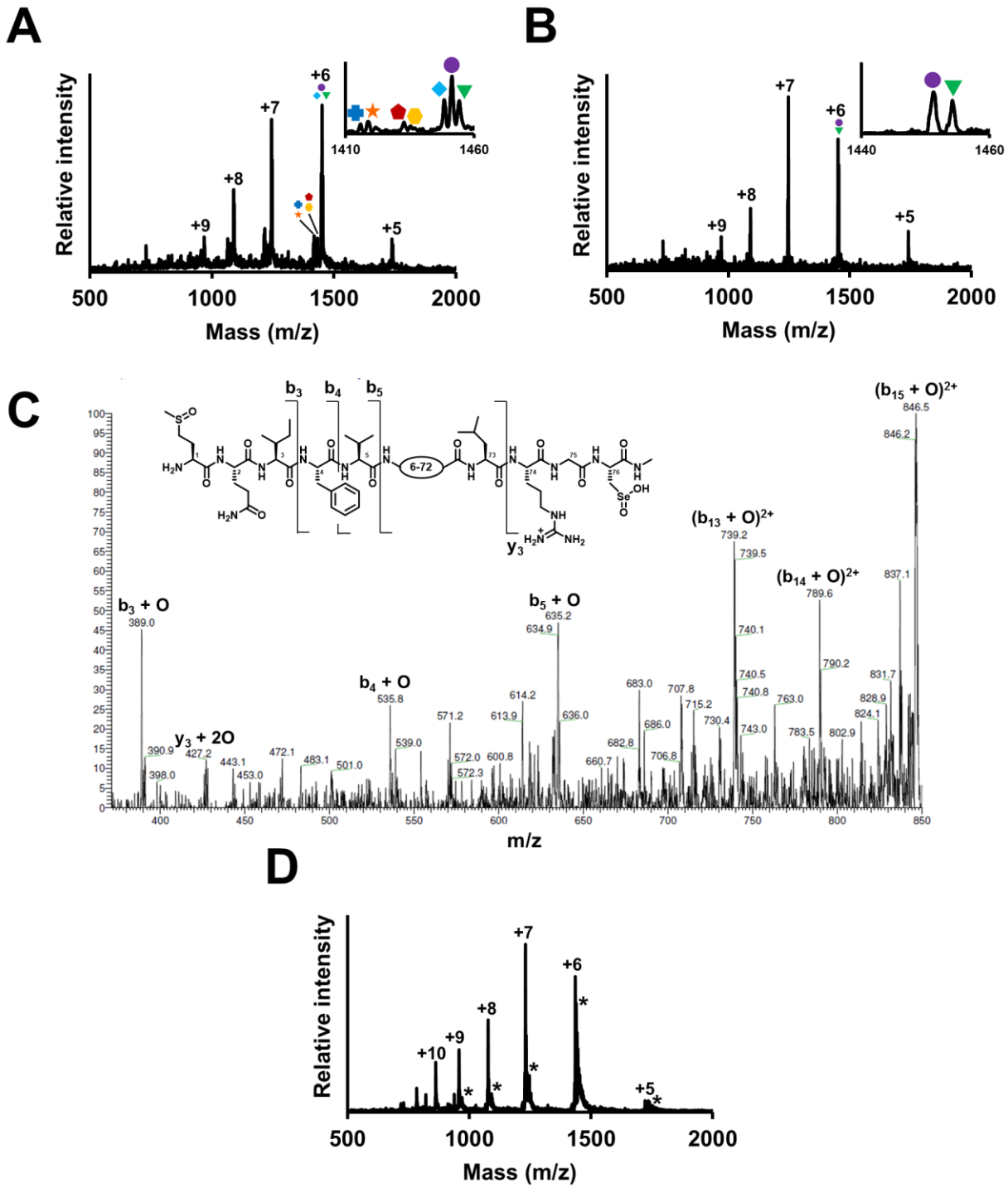


Figure S2. **Oxidation products of Ub-SeCys-*N*-methylamide 3.** (**A**) ESI-MS of oxidation products: **3**[O_3] (green triangle), observed [M+H$^+$] 8,720.9 ± 1.5 Da. Calcd. 8,719.8 Da. **3**[O_2] (purple circle), observed [M+H$^+$] 8,703.3 ± 1.5 Da. Calcd. 8,703.8 Da. **3**[O_1] (light blue diamond), observed [M+H$^+$] 8,685.7 ± 2.0 Da. Calcd. 8,687.8 Da. Ub-Dha-*N*-methylamide (**4**) [O_1] (yellow hexagon), observed [M+H$^+$] 8,607.0 ± 1.9 Da. Calcd. 8,606.8 Da. **4** (red pentagon), observed [M+H$^+$] 8,591.0 ± 1.6 Da. Calcd. 8,590.8 Da. Hydrolyzed Ub(1-75)-CO_2H (blue cross), observed [M+H$^+$] 8,508.8 ± 1.5 Da. Calcd. 8507.8 Da. Hydrolyzed Ub(1-75) [O_1] (orange star), observed [M+H$^+$] 8,525.4 ± 2.2 Da. Calcd. 8,523.7 Da. All reported masses are isotopic averages. (**B**) ESI-MS of oxidation products: **3**[O_3] (green triangle), observed [M+H$^+$] 8,719.1 ± 6.1 Da. Calcd. 8,719.8 Da. **3**[O_2] (purple circle), observed [M+H$^+$] 8,703.4 ± 2.9 Da. Calcd. 8,703.8 Da. All reported masses are isotopic averages. (**C**) ESI-MS-MS of **3**[O_3]. All reported masses are

monoisotopic. (**D**) **4**[O_1] (yellow hexagon), observed [M+H^+] 8,609.6 ± 2.7 Da. Calcd. 8,606.8 Da. Asterisks (*) indicate Na^+ adducts. All reported masses are isotopic averages.

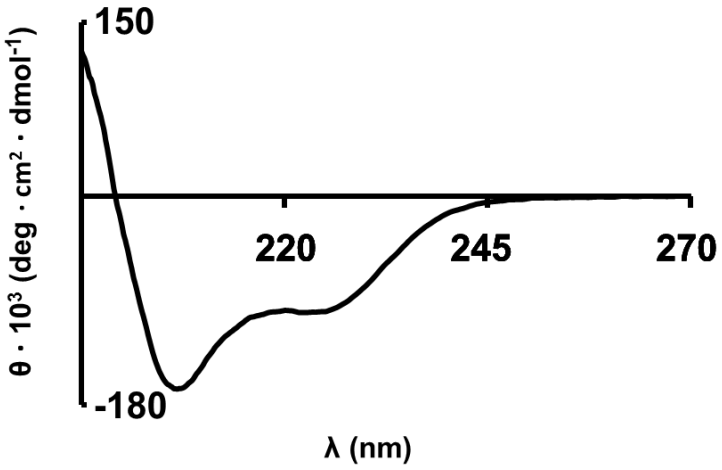


Figure S3. Circular Dichroism (CD) spectrum of Ub-Dha-*N*-methylamide 4.

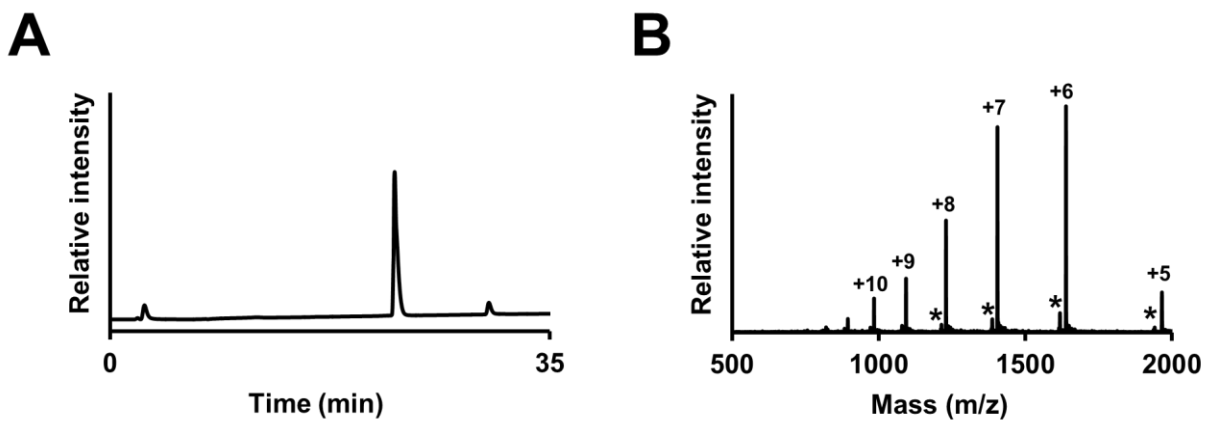


Figure S4. **Purification of FLAG-Ub(1-75)-MESNa α-thioester.** (**A**) C18 Analytical RP-HPLC chromatogram of purified FLAG-Ub(1-75)-MESNa α-thioester using a gradient of 0-73% B over 30 min. (**B**) ESI-MS of purified FLAG-Ub(1-75)-MESNa α-thioester, observed [$M+H^+$] 9,830.9 ± 2.5 Da. Calcd. 9,830.3 Da. Asterisks indicate hydrolysis product FLAG-Ub(1-75)-CO_2H, observed [$M+H^+$] 9,706.6 ± 2.0 Da. Calcd. 9,705.0 Da. All reported masses are isotopic averages.

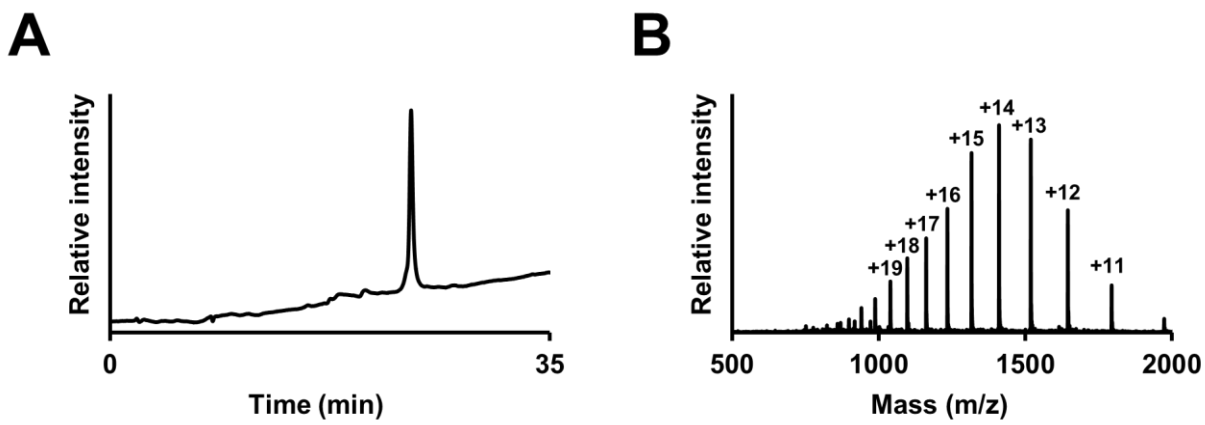
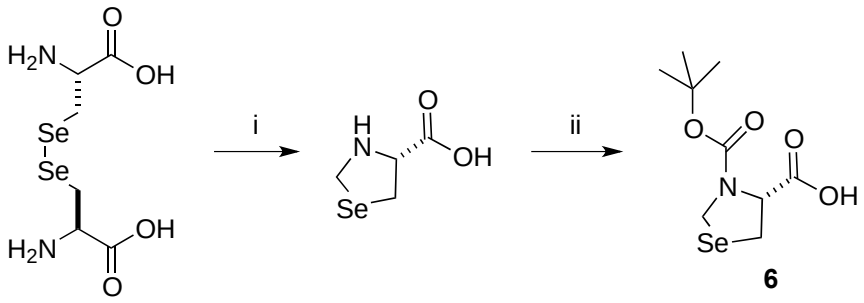


Figure S5. **Purification of FLAG-Ub-Gly76Sec-*N*-methylamide.** (**A**) C18 Analytical RP-HPLC chromatogram of purified FLAG-Ub-Gly76Sec-*N*-methylamide using a gradient of 0-73% B over 30 min. (**B**) ESI-MS of purified dimeric FLAG-Ub-Gly76Sec-*N*-methylamide, observed $[M+H^+]$ 19,738.0 ± 3.5 Da. Calcd. 19,736.1 Da. All reported masses are isotopic averages.



Scheme S1. Synthesis of N$^{\alpha}$-Boc-L-selenazolidine 6. (i) (1) $NaBH_4$, NaOH, H_2O-EtOH, 25 ^{o}C. (2) AcOH, HCHO, 4 ^{o}C, 87%. (ii) Boc_2O, DIEA, H_2O-MeOH, on ice, 39%.

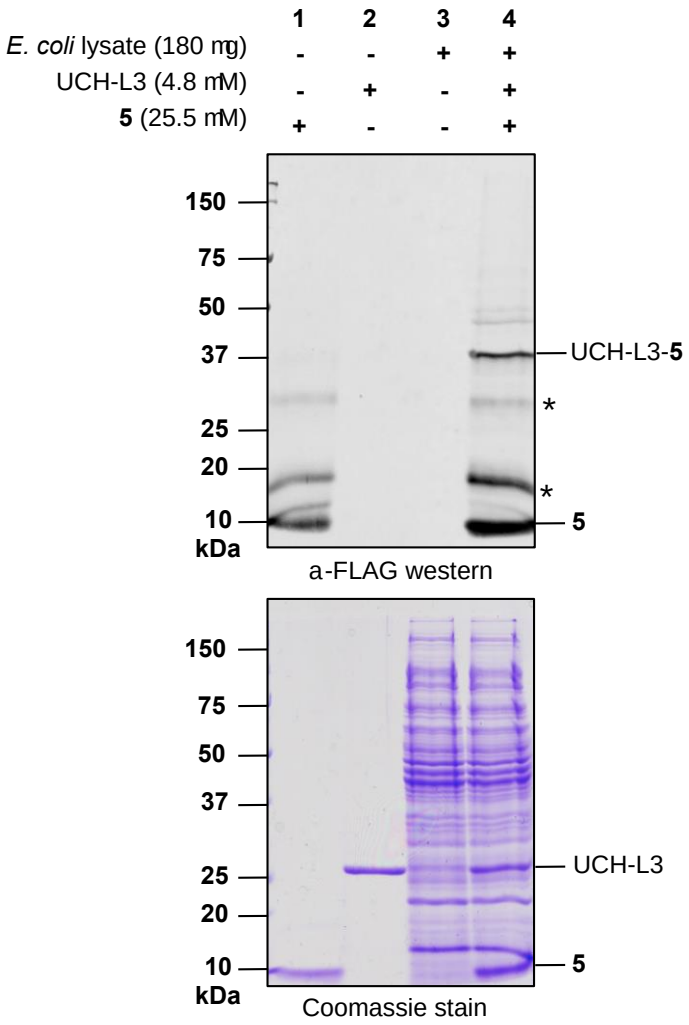


Figure S6. Labeling of purified UCH-L3 added to an *E. coli* cell lysate. Western Blot of *E. coli* lysate containing a known amount of purified wild-type UCH-L3 incubated with **5** for 12 h at 37 °C. The covalent UCH-L3-**5** adduct was detected with an anti-FLAG antibody (F3165, Sigma-Aldrich) (Top). Coomassie-stained 10% SDS-PAGE of samples probed by western blot (bottom). Asterisks indicate oligomers of **5**.

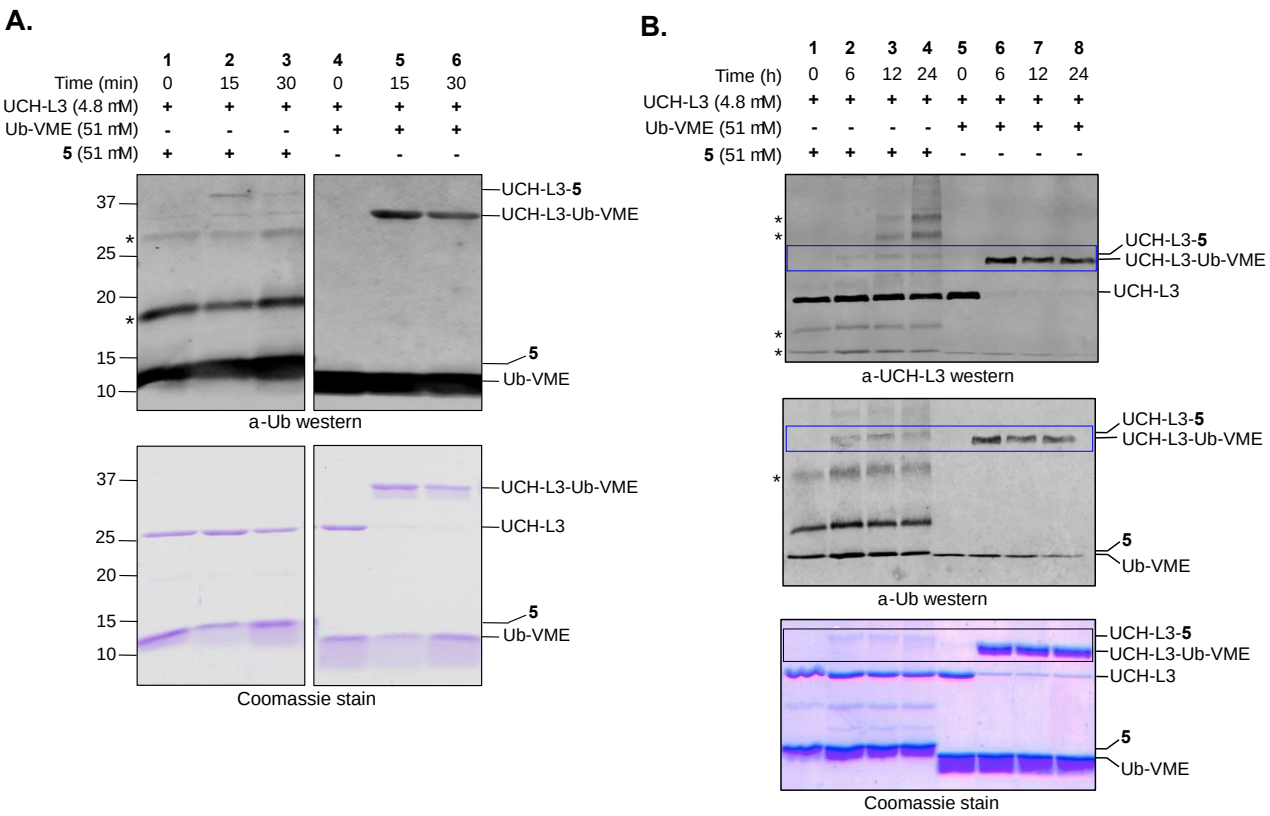


Figure S7. Kinetics of UCH-L3 labeling with synthetic FLAG-Ub-Dha (5) and commercially sourced Ub-VME. (A) Early time-points of the labeling reaction between UCH-L3 and the indicated probes visualized by western blot with an anti-Ub antibody (P4D1, Santa Cruz Biotech). **(B)** Extended time-points of the labeling reaction between UCH-L3 and the indicated probes visualized by western blot with an anti-Ub antibody (P4D1, Santa Cruz Biotech). Asterisks indicate non-specific bands.

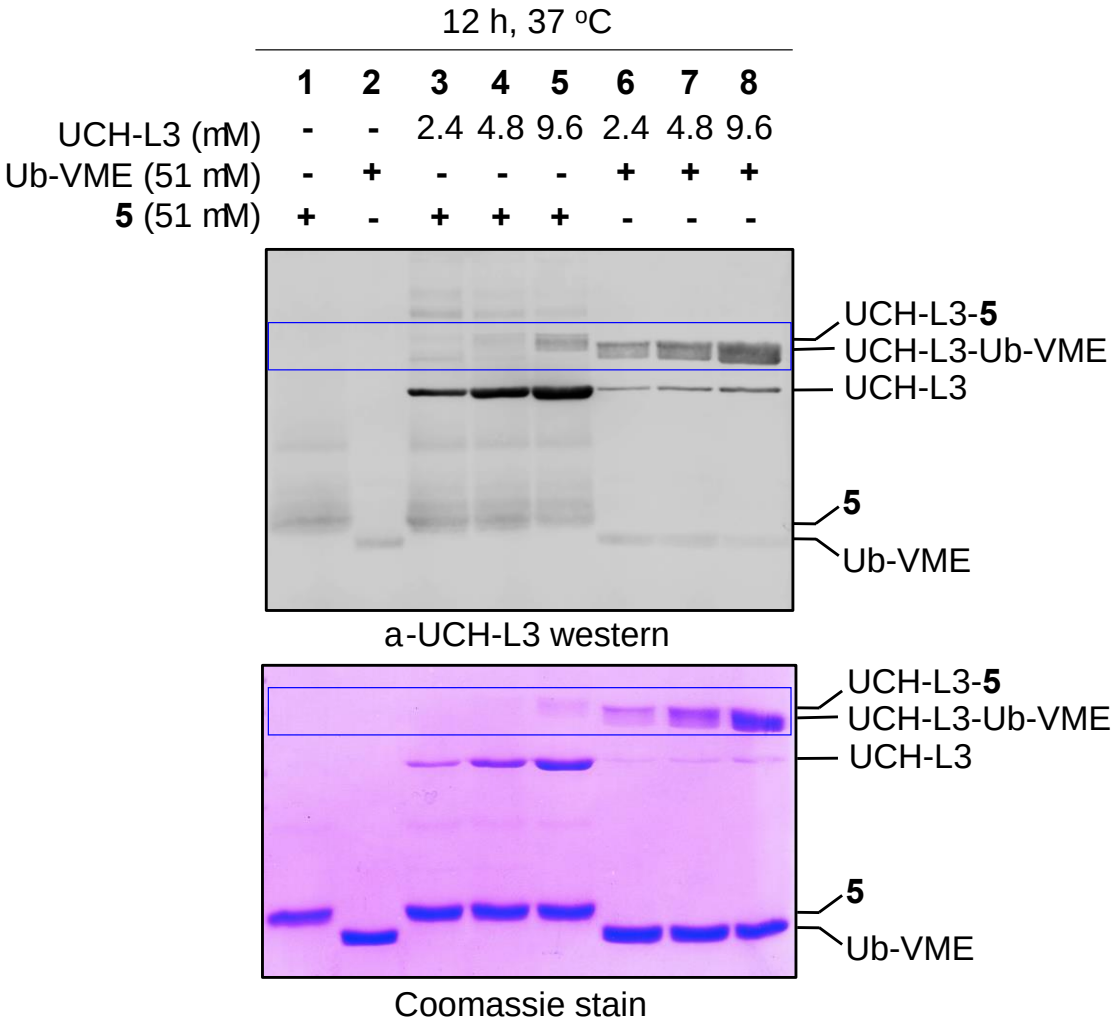


Figure S8. Concentration dependence of UCH-L3 labeling with synthetic FLAG-Ub-Dha (5) and commercially sourced Ub-VME. Western blot of 12 h time-point in labeling assays with increasing concentrations of UCH-L3 and fixed concentrations of the indicated probes visualized with an anti-UCH-L3 antibody (Cell Signaling Technologies #8141) (Top). Coomassie-stained 12% SDS-PAGE of samples probed in western blots (Bottom).

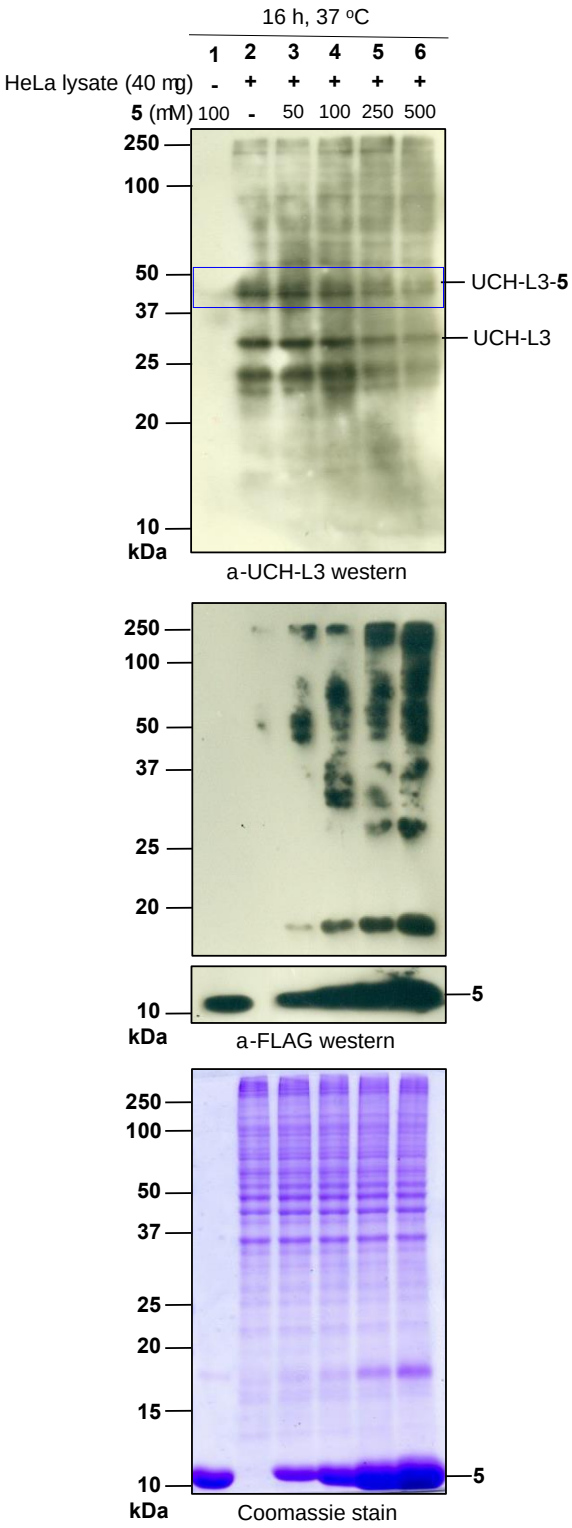


Figure S9. Labeling of endogenous proteins in HeLa whole-cell lysates with FLAG-Ub-Dha (5). Western blot of UCH-L3 labeling in HeLa cell lysates with increasing concentrations of **5** using an anti-UCH-L3 antibody (Cell Signaling Technologies #8141) (Top). Western blot of HeLa proteome labeling with increasing concentrations of **5** using an anti-FLAG antibody (F3165, Sigma-Aldrich) (Middle). Coomassie-stained 12% SDS-PAGE of samples probed in western blots (Bottom).

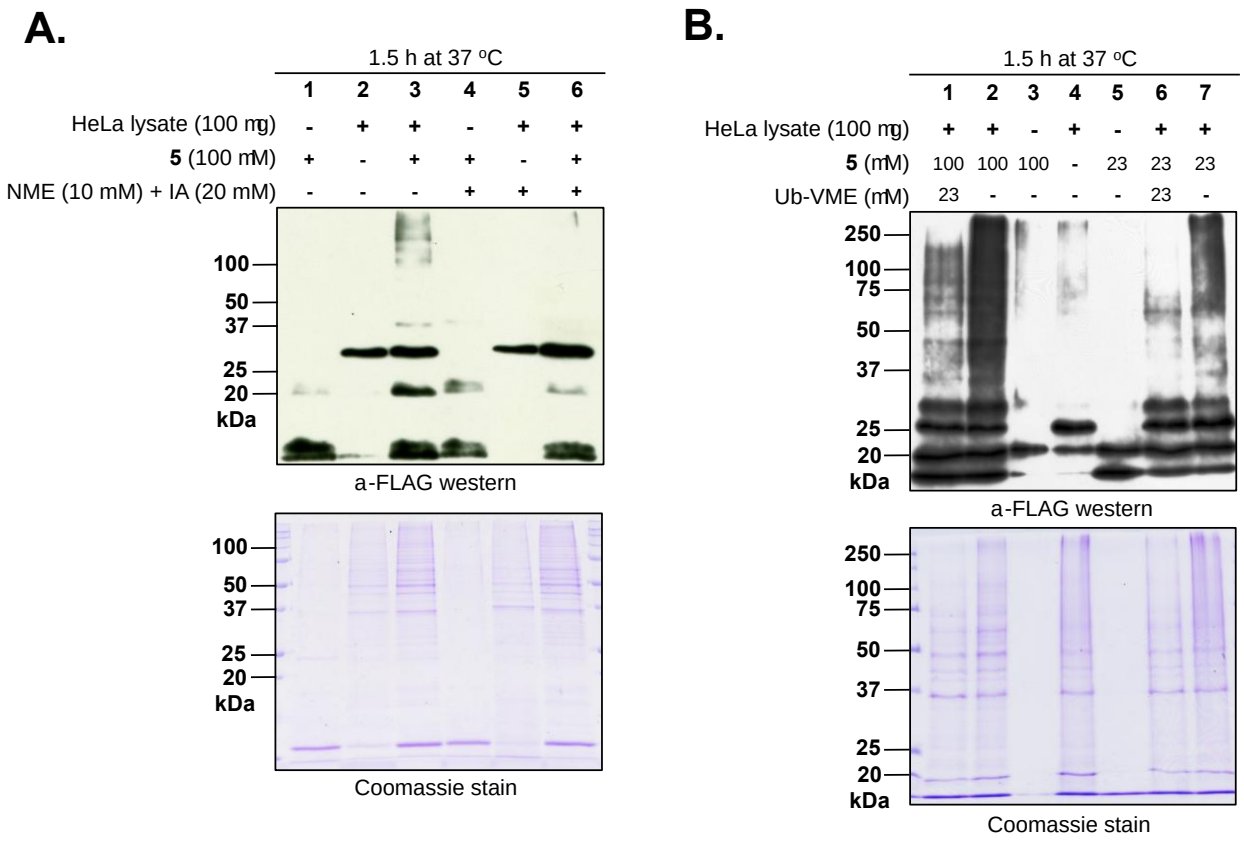


Figure S10. Competing activities of FLAG-Ub-Dha (5) and DUB inhibitors. (**A**) Western blot of HeLa proteome labeling by **5** in the presence or absence of the DUB inhibitors N-Ethylmaleimide (NEM) and 2-Iodoacetamide (IA) using an anti-FLAG antibody (F2555, Sigma-Aldrich). A coomassie-stained loading control is shown at bottom. (**B**) Western blot of HeLa proteome labeling by **5** in the presence or absence of the DUB labeling probe Ub-VME using an anti-FLAG antibody (ab1162, Abcam). A coomassie-stained loading control is shown at bottom.

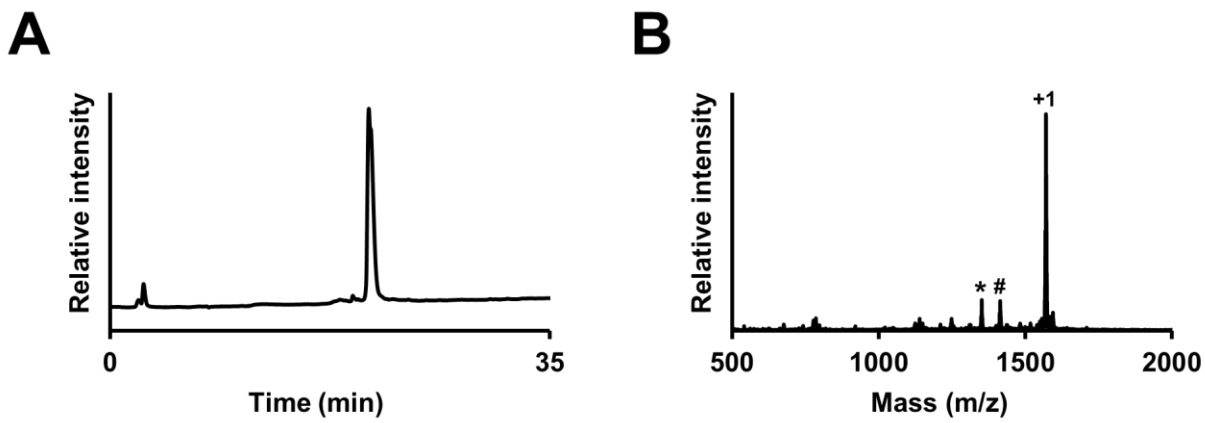


Figure S11. Purification of TRIM25(112-124) K117-iso-selenazolidine. (**A**) C18 Analytical RP-HPLC chromatogram of purified TRIM25(112-124) K117-iso-selenazolidine using a gradient of 0-73% B over 30 min. (**B**) ESI-MS of purified TRIM25(112-124) K117-iso-selenazolidine, observed [$M+H^+$] 1,569.8 Da. Calcd. 1,569.8 Da. Fragments observed during MS are: b12 ion (asterisk), observed [M^+] 1,350.8 Da. Calcd. 1350.5 Da. Singly oxidized y13 ion (hashtag), observed [M^+] 1,413.7 Da. Calcd. 1,414.6 Da. All reported masses are isotopic averages.

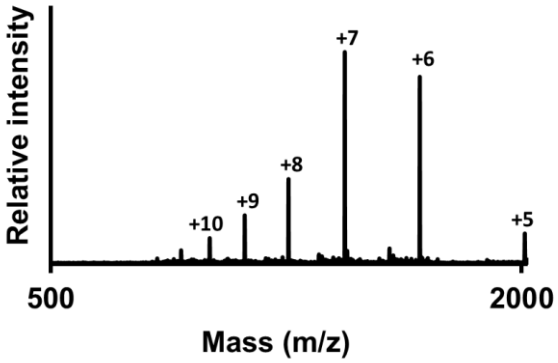


Figure S12. ESI-MS of purified Ub-SeCys-TRIM25 8. Observed [M+H$^+$] 10,047.8 ± 2.4 Da, calcd. 10,047.5 Da. Reported masses are isotopic averages.

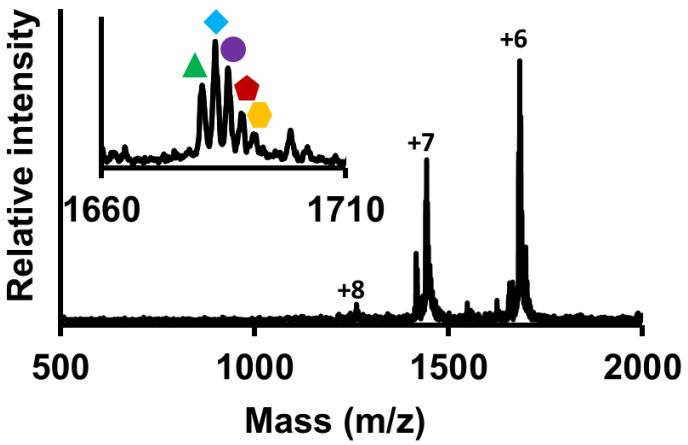


Figure S13. ESI-MS of oxidized Ub-Sec-TRIM-25 8. **8**[O_2] (green triangle), observed [M+H^+] 10,079.4 ± 1.6 Da. Calcd. 10,078.6 Da. **8**[O_3] (blue diamond), observed [M+H^+] 10,094.5 ± 2.1 Da. Calcd. 10,094.6 Da. **8**[O_4] (purple circle), observed [M+H^+] 10,110.6 ± 1.3 Da. Calcd. 10,110.2 Da. **8**[O_5] (red pentagon), observed [M+H^+] 10,126.8 Da ± 0.8. Calcd 10,126.6 Da. **8**[O_6] (yellow hexagon), observed [M+H^+] 10,142.1 ± 1.2 Da. Calcd 10,142.6 Da. All reported masses are isotopic averages.

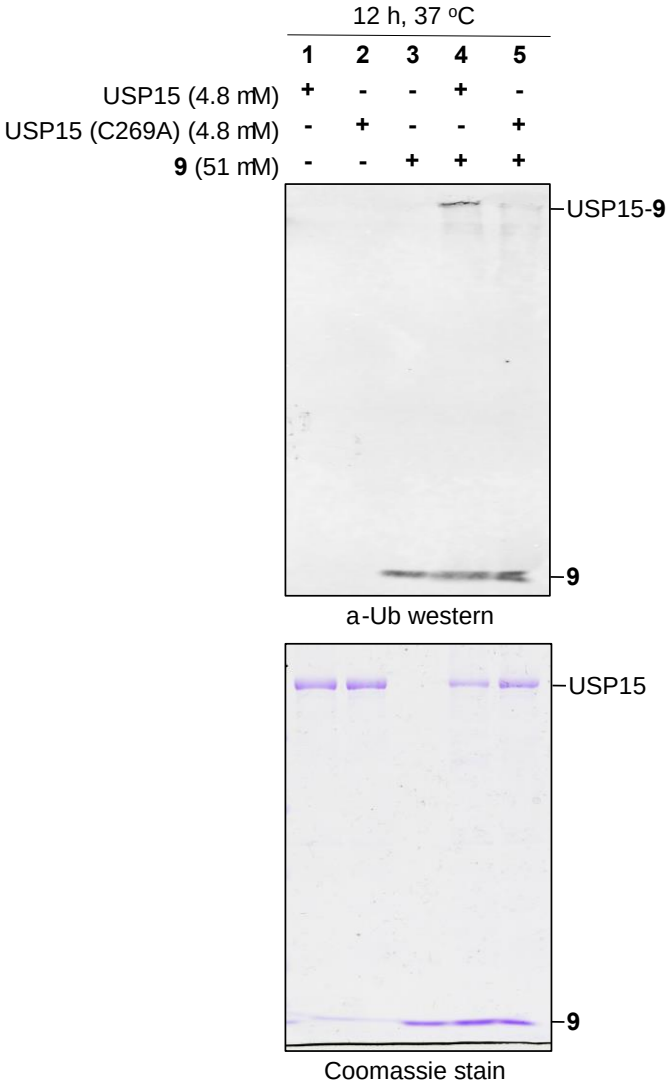


Figure S14. Labeling of the Usp15 active site Cys269 by Ub-Dha-TRIM-25 (9). Labeling of full-length Usp15 and the Usp15 C269A mutant by probe **9** followed by western blot using an anti-Ub antibody (P4D1, Santa Cruz Biotech) (Top). Coomassie-stained 8% gel of samples probed in western blots (Bottom).

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