Supporting Information

Facile preparation of UFMylation Activity-Based Probes by Chemoselective Installation of Electrophiles at the C-terminus of Recombinant UFM1

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# Table of Contents

1. MATERIALS ........................................................................................................................ 4
   1.1 SAFETY STATEMENT ............................................................................................................. 4  
   1.2 GENERAL REAGENT INFORMATION ......................................................................................... 4  
   1.3 GEL ELECTROPHORESIS ........................................................................................................ 4  
   1.4 REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) ........................ 5  
   1.5 WESTERN BLOTTING ............................................................................................................. 5  
   1.6 PROTEIN AND SMALL MOLECULES CHARACTERIZATION .......................................................... 5  
   1.7 CD-MEASUREMENTS ............................................................................................................. 6  
   1.8 GENE CONSTRUCTS .............................................................................................................. 6  
   1.9 RECOMBINANT PROTEINS ...................................................................................................... 7  
   1.10 CELL CULTURE AND PREPARATION OF CELL LYSATES ........................................................... 8

2. METHODS ........................................................................................................................... 8
   2.1 PROCEDURE FOR HYDRAZINOLYSIS OF UFM1 ................................................................. 8  
   2.2 PREPARATION OF SYMMETRICAL ANHYDRIDES OF CARBOXYLIC ACIDS ......................... 9  
      2.2.1 GLYCIDIC ACID SYNTHESIS ................................................................................................. 9  
      2.2.2 GENERAL PROCEDURE FOR SYNTHESIS OF ANHYDRIDES 10, 11, 12 ............................. 10  
      2.3 PROCEDURE FOR UFM1-HYDRAZIDE MODIFICATION ....................................................... 10  
   2.4 STABILITY TESTS FOR UFM1 PROBE 2 .................................................................................. 10  
   2.5 PROBES USED FOR IN VITRO CROSSLINKING ASSAYS ....................................................... 11  
      2.5.1 CROSSLINKING WITH E1S ................................................................................................. 11  
      2.5.2 CROSSLINKING WITH E2S ................................................................................................. 11  
      2.5.3 CROSSLINKING WITH DUBS ............................................................................................... 11  
      2.5.4 CROSSLINKING OF PROBE 2 WITH UFSP2, DIGESTION AND MS/MS ANALYSIS .......... 11  
   2.6 CROSSLINKING OF PROBES IN LYSATES ............................................................................. 12  
      2.6.1 LABELING OF ENDOGENOUS ENZYMES IN CELL LYSATES ............................................. 12  
      2.6.2 LABELING OF SPIKED UFSP2 IN CELL LYSATES ............................................................. 12  
   2.7 KNOCKDOWN AND KNOCKOUT CELL LINE GENERATION ............................................... 12  
   2.8 ELECTROPORATION EXPERIMENTS ...................................................................................... 13  
   2.9 IMMUNOPRECIPITATION ON ANTI-FLAG BEADS .............................................................. 13  
   2.10 ON-BEAD DIGESTION ......................................................................................................... 13

3. SUPPLEMENTARY FIGURES .......................................................................................... 14
   3.1 ANALYTICAL DATA FOR UFM1 C-TERMINAL PROBES ......................................................... 14  
      3.1.1 ANALYTICAL DATA FOR MODIFIED UFM1 HYDRAZIDE 1 .............................................. 14  
      3.1.2 ANALYTICAL DATA FOR MODIFIED UFM1 PROBE 2 ...................................................... 15  
      3.1.3 ANALYTICAL DATA FOR MODIFIED UFM1 PROBE 3 ...................................................... 16  
      3.1.4 ANALYTICAL DATA FOR MODIFIED UFM1 PROBE 4 ...................................................... 17  
      3.1.5 ANALYTICAL DATA FOR MODIFIED UFM1 PROBE 5 ...................................................... 18  
      3.1.6 ANALYTICAL DATA FOR MODIFIED UFM1 PROBE 6 ...................................................... 19
3.2 STABILITY TESTS OF UFM1 PROBE 2 ................................................................. 20
3.3 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH E1S, E2S, DUBS ........ 21
3.3.1 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH UBA1 AND UBA5 ...... 21
3.3.2 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH A PANEL OF E2S ...... 21
3.3.3 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH UFC1 .................... 22
3.3.4 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH A PANEL OF DUBS .... 23
3.3.5 SDS-PAGE ANALYSIS OF UFM1 PROBES IN VITRO CROSSLINKING WITH UFSP2 ................. 23
3.4 WESTERN BLOTS OF UFM1 C-TERMINAL PROBES INCUBATED WITH LYSATES ................ 24
3.4.1 WESTERN BLOTS OF UFM1 PROBES 2 – 6 INCUBATED WITH HCT116 AND HCT116 UFM1 KD WHOLE CELL LYSETS ........................................................................ 24
3.4.2 WESTERN BLOTS OF UFM1 PROBES 2 – 6 INCUBATED WITH SPIKED UFSP2 IN HEK293T WHOLE CELL LYSATES ......................................................................................... 25
3.4.3 WESTERN BLOTS OF UFM1 PROBES 2, 6 NUCLEOFECTED INTO HEK293T UFM1 KD AND HEK293T UFM1 KD, UFSP2 KO CELL LINES ........................................................................ 25

4. SUPPLEMENTARY TABLES .................................................................................. 26

4.1 PRIMERS, TEMPLATES, AND PCR METHODS USED FOR MOLECULAR CLONING ............. 26
4.2 PROTEIN SEQUENCES .................................................................................. 26

5. SUPPLEMENTARY REFERENCES ...................................................................... 29
1. MATERIALS

1.1 Safety statement
No unexpected or unusually high safety hazards were encountered.

1.2 General reagent information
Chemical reagents were purchased from Sigma Aldrich (Buchs, Switzerland), Acros Organics (Geel, Belgium), TCI Europe (Zwijndrecht, Belgium) and were used without further purification. CH$_2$Cl$_2$, DMF, EtOH and MeOH were purchased from Fisher Scientific (Geel, Belgium) and Sigma Aldrich (Buchs, Switzerland) and used without further purification (reagent or HPLC grade). Milli-Q water was obtained from a Millipore purification system. Q5 High-Fidelity DNA Polymerase, PCR reagents, Gibson Assembly Master Mix, were purchased from New England BioLabs (Ipswich, MA, USA). DNase I was obtained from Roche Diagnostics GmbH (Mannheim, Germany). DNA purification kits were purchased from Fisher Scientific (Geel, Belgium) and Zymo Research (Irvine, CA, USA). Lysozyme (22500 U/mg) was obtained from Axon Lab AG (Baden, Switzerland). Kanamycin sulfate and ampicillin were obtained from AppliChem GmbH (Darmstadt, Germany). Ni-NTA agarose resin was obtained from Qiagen GmbH (Hilden, Germany). Bradford protein assays were performed using Protein Assay Dye Reagent Concentrate from Bio-Rad. Dialysis tubing was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Amicon Ultra centrifugal filters were purchased from Merck (Darmstadt, Germany), VivaSpin 500 centrifugal concentrators were purchased from Satorius Stedim Lab (Stonehoude, UK), PD MiniTrap desalting columns were purchased from GE Healthcare. All buffers were prepared using Mili-Q water, sterile filtered (0.2 μm membrane filter), pH was adjusted at the temperature the buffer was used. Oligonucleotide synthesis and sequencing was carried out by Microsynth AG (Balgach, Switzerland). Synthetic genes were ordered from Thermo Fisher Scientific (Waltham, MA, USA) and GenScript Biotech (New Jersey, USA). Pierce Anti-DYKDDDDK Magnetic Agarose Beads were purchased from Sigma Aldrich (St. Louis, USA). 100 μL and 10 μL Pierce™ C18 Tips were purchased from Thermo Fisher Scientific. Pierce Trypsin Protease was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell culture reagents including Dulbecco’s modified eagle medium (DMEM), phosphate-buffered saline (PBS), trypsin-EDTA, blasticidin, Lipoectamine were purchased from Sigma Aldrich. Fetal Bovine Serum (FBS) was purchased from Thermo Fisher Scientific.

1.3 Gel electrophoresis
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-PROTEAN Tetra Cell system (Bio-Rad) connected to the PowerPac Basic (Bio-Rad).
$\text{power supply. Reducing samples were treated with sample buffer (Laemmli 2x or 4x Concentrate, Sigma Aldrich), heated to 95 °C for 5 min and used for separation. A 10-180 kDa pre-stained protein ladder (Thermo Fisher) was applied to at least one well of each gel (3 μL). Samples were separated on 8-16% gradient Mini-PROTEAN TGX Precast gels (Bio-Rad) for 30 minutes at 200 V. Gels were stained with Coomassie (0.1% Coomassie Brilliant Blue R, 40% MeOH, 10% acetic acid) with agitation for 1 h and subsequent destaining (40% MeOH, 10% acetic acid). Destained gels were imaged on a Bio-Rad Molecular Imager Pharos FX (Coomassie Blue). Gel images are shown in Supplementary figures 7 – 11. Data was illustrated using Adobe Illustrator.}$

1.4 Reverse-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC was carried out on Jasco analytical instrument with simultaneous monitoring of the eluent at 220 nm, 254 nm, and 301 nm at room temperature (rt). The mobile phase was Milli-Q water with 0.1% TFA (solvent A) and HPLC-grade MeCN with 0.1% TFA (solvent B). Shiseido C18 (5 μL, 4.6 mm I.D. x 250 mm) column or Agilent Eclipse XDB-C8 (5 μm, 4.6 mm x 150 mm) at a flow rate of 1 mL/min, was used with the following method: 20% solvent B for 3 min, 20 to 95% solvent B over 14 min, and 95% solvent B for 3 min.

1.5 Western Blotting

Samples were separated using SDS-PAGE on a 8-16% gradient Mini-PROTEAN TGX Precast gels (Bio-Rad) for 30 minutes at 200 V. Proteins were electrophoretically transferred onto PVDF membrane (Bio-Rad) using Mixed MW method (1.3 A, up to 25 V, 7 minutes) and blocked using 5 wt% skim milk in TBS-T for 1 h at rt. Membrane was washed using TBS-T (3 x 5 minutes) and incubated with the primary antibody: anti – FLAG (Invitrogen, MA1-91878, 1:1000), anti – UFM1 (Invitrogen, PA5-103016, 1:1000) or anti – UFSP2 (Santa Cruz Biotechnology, sc-376084, 1:100), in TBS-T (1 wt% BSA) for 1 h at rt. After washing (3 x 5 minutes) the membrane was incubated with HRP-linked secondary antibody anti – Mouse (Invitrogen, 62-6520, 1:2000) or anti – Rabbit (Cell Signalling, # 7074, 1:3000) for 18 h at 4 °C. The membrane was washed with TBS-T (3 x 5 minutes). Bands were visualized with a chemiluminescent reagent (Clarity Western ECL, Bio-Rad) on a Bio-Rad Molecular Imager Pharos FX (Chemiluminescence). Images are shown in Supplementary figures 12 – 14. Data was illustrated using Adobe Illustrator.

1.6 Protein and small molecules characterization

High-resolution mass spectra were obtained at the Molecular and Biomolecular Analysis Service (MoBiAS) of the ETH Zürich Department of Chemistry and Applied Biosciences on a
Bruker Daltonics maXis ESI-QTOF spectrometer (ESI) or a Bruker Daltonics solariX spectrometer (MALDI-FTICR-MS) using α-cyano-4-hydroxycinnamic acid as matrix. NMR spectra were recorded on a Bruker AV-400 spectrometer. Chemical shifts are given in ppm relative to residual solvent peaks. Tandem MS experiments were performed using ESI-TIMS-QTOF-MS system (TimsTOF Pro, Bruker Daltonics, Germany) with collision-induced dissociation (CID) and N₂ as the collision gas. Tryptic peptides were pressure loaded onto a reversed phase 25 cm x 75 μm i.d. C18 1.6 μm column (Ionoptics Ltd., Australia) with a reversed phase 5 mm x 0.3 mm i.d. C18 5 μm column (Thermo Scientific, Lithuania) as guard column at 40 °C. The mobile phase consisted of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient started at 2% of B and was linearly increased to 35% in 120 min at flowrate of 300 μl min⁻¹. A second gradient profile, started at 35% of B and was linearly increased to 95% in 2 min at flowrate of 300 μl min⁻¹. Followed by isocratic conditions of 95% B at flowrate of 300 μl min⁻¹ for 8 min. Total run time, including the conditioning of the column to the initial conditions, was 163 min. Further data processing was performed with Data Analysis 5.3 software (Bruker Daltonics, Germany) using a processing script to generate export files and reports. Advanced raw data processing for bioanalytical interpretations were performed using PEAKS Studio software (Bioinformatics Solutions Inc., Canada).

1.7 CD-measurements
Retention of UFM1 structural integrity was confirmed by circular dichroism spectroscopy (CD). UFM1-hydrazide 1 and UFM1-chloroacetyl 2 were buffer exchanged to PBS and diluted to approximately 20 μM final concentration. Circular dichroism was measured using a JASCO J-1500 Spectrometer at 20 °C. CD spectra were recorded ranging from 300 to 180 nm at a scan rate of 20 nm per minute and a scan width of 0.5 nm using a quartz cuvette with a 1 mm path length. Measurements were plotted using Graphpad PRISM.

1.8 Gene constructs
Human UFM1 (UniProt P61960), UFC1 (UniProt Q9Y3C8) and UBA5 (UniProt Q9GZZ9) genes were codon optimized for E. coli expression and ordered from Thermo Fisher Scientific. UFM1 was subcloned into FLAG-MxE-GyrA-His₈ pET28 vector (Merck Novagen) using Gibson assembly (New England BioLabs). UFC1 was subcloned into His₈-TEV pET28 vector using Gibson assembly. UBA5 was subcloned into His₈-SUMO2 pET28 vector using Gibson assembly. The cDNA for human UFSP2 (UniProt Q9NUQ7) was ordered from GenScript (ID OHu01932, #NM_018359.5) and subcloned into His₈-SUMO2 pET28 vector using Gibson assembly. Human USP21 (UniProt Q9UK80) in pOPINS was a gift from David Komander.
(Addgene plasmid #61585); human SENP1 (UniProt Q9P0U3) and human SENP8 (UniProt Q96LD8) in pET28a were gifts from Guy Salvesen (Addgene plasmid #16356, #16361); yeast YUH1 (UniProt P35127) in pET3a was a gift from Cecile Pickart (Addgene plasmid #18895). Human UBA1 (UniProt P22314) in pET28 was a gift from Gaku Akimoto. Human UBE2K (UniProt P61086) in pET3d was a gift from Cecile Pickart (Addgene plasmid #18892); human UBE2B (UniProt P63146), UBE2L3 (UniProt P68036) and UBC12 (UniProt P61081) in pDEST17 were gifts from Wade Harper (Addgene plasmid #15781, #15795, #15798); human UBC9 (UniProt P63279) in pET28 was a gift from Raphael Hofmann. If not noted otherwise all genes were cloned into His6-TEV pET28 vector using Gibson assembly. Variants of UFC1 (C116A) and UFSP2 (C302A) were prepared using primers, templates, and PCR methods (Q5 protocol by New England BioLabs) listed in Supplementary Table 1. A list of constructs and protein sequences is given in Supplementary Table 2. All constructs were verified by DNA Sanger sequencing.

1.9 Recombinant proteins
Recombinant E1s, E2s and DUBs were expressed in E. coli and purified following standard procedures with minor changes.1,2 In brief, chemically competent BL21 (DE3) cells or BL21-CodonPlus (DE3)-RIL (for UBA5 and UFSP2) were heat-shock transformed with the plasmids and single colonies were used to inoculate overnight precultures in selective lysogeny broth (LB) Miller medium (UFM1, UFC1, UBE2K, UBE2B, UBE2L3, UBC12, UBC9, USP21, SENP8, SENP1 and YUH1). Following 1:1000 dilution with fresh selective LB Miller medium cultures were grown in baffled shake flasks at 37 °C until an OD600 of 0.6 was reached. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.5 mM. Expressions were carried out for 18 h at 25 °C or 8 h at 25 °C. Alternatively, for UBA5, UFSP2 and UBA1 autoinduction medium was used.3 Overnight precultures were diluted 1:1000 and cells were grown overnight at 37 °C. Cultures were grown in a baffled shake flasks at 37 °C until an OD600 of 6 was reached and then at 16 °C for 48 h. Cells were collected by centrifugation (5,000 x g, 25 min, 4 °C), resuspended in lysis buffer (20 mL per liter cell culture for LB and 50 mL per liter culture for autoinduction medium), 50 mM Tris, 200 mM NaCl, 10% glycerol pH 7.4 (UFM1, UFC1, UBE2K, UBE2B, UBE2L3, UBC12, UBC9, USP21, SENP8, SENP1 and YUH1), PBS pH 7.4 (UBA5 and UFSP2) or 50 mM HEPES, 350 mM NaCl pH 7.8 (UBA1) and stored at -80 °C until further purification. Cell suspensions were thawed at room temperature and placed on ice, supplemented with lysozyme (20 μg/mL) and DNase I (0.1 mg/mL) for UFM1-GyrA; 1 mM DTT, lysozyme (20 μg/mL) and DNase I (0.1 mg/mL) for UFC1, UBA5, UFSP2, UBE2K, UBE2B, UBE2L3, UBC12, UBC9, USP21, SENP8, SENP1, YUH1, UBA1 and incubated on a nutating mixer at 4 °C for
30 minutes. Cells were lysed by sonication and the suspensions were cleared by centrifugation (30,000 x g, 45 min, 4 °C) and filtration (0.2 μm membrane filter). Supernatants were subjected to gravity Ni-NTA affinity purification using binding and eluting buffers (50 mM Tris, 200 mM NaCl, 30 mM and 300 imidazole pH 7.2 for UFM1-Mxe GyrA; 50 mM Tris, 200 mM NaCl, 30 mM and 300 imidazole, 1mM DTT, pH 7.4 for UFC1, UBE2K, UBE2B, UBE2L3, UBC12, UBC9, USP21, SENP8, SENP1 and YUH1; PBS with 30 mM and 300 mM imidazole, 1 mM DTT pH 7.4 for UBA5 and UFSP2; 50 mM HEPES, 350 mM NaCl pH 7.8 with 20 mM and 300 mM imidazole for UBA1). UFM1 was subject to further intein cleavage without additional purification. UFC1, UBA5 and UFSP2 were subject to further purification by anion exchange chromatography (Mono Q 5/50 GL). Samples were dialyzed against buffer A (25 mM Tris, 25 mM NaCl, 1 mM DTT pH 8.5) and purified with buffer A and a gradient of buffer B (25 mM Tris, 1 M NaCl, 1 mM DTT pH 8.5). His-tag was removed by TEV (tobacco etch virus) protease in 50 mM Tris, 200 mM NaCl, 1mM DTT, pH 8.0. For UBA5 and UFSP2, expressed as a fusion with SUMO, SENP1 (1 wt% relative to the protein concentration) was added and left on a nutating mixer at 4 °C overnight. UBA1, UBE2K, UBE2B, UBE2L3, UBC12, UBC9, USP21, SENP8, SENP1 and YUH1 were purified with ion exchange chromatography as has been previously reported.

1.10 Cell culture and preparation of cell lysates
HEK293T and HCT116 cell lines used in the study originated from ATCC. HCT116 UFM1 KD, HEK293T UFM1 KD, HEK293T UFSP2 KO were generated using CRISPR-Cas9, conditions given in Supplementary Information 2.6. Cell lines were cultured under standard conditions at 37 °C with 5% CO₂ atmosphere in DMEM (15-013-CV) supplemented with 10% (v/v) fetal bovine serum (FBS, Omega Scientific, FB-11, Lot no. 441224) and penicillin-streptomycin. Once grown to 90% confluence, cells were scraped off the flask in PBS, pelleted by centrifugation (500 g, 5 min, rt) and resuspended into 4 x pellet volume of ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 1 vol% NP-40, 1 mM TCEP, and a 100x of Protease inhibitor mix). Further sonicated on ice for 2 x 10 sec, passed through a 25-gauge needle 5-times and clarified by centrifugation (15,000 g, 30 min, 4 °C). Lysate concentrations were estimated using BCA method, lysates were aliquoted, flash frozen and stored at -80 °C until further usage.

2. METHODS
2.1 Procedure for hydrazinolysis of UFM1
FLAG-UFM1-Mxe GyrA-His6 fusion protein, 200 μM, was dialyzed against reaction buffer (50 mM Tris, 200 mM NaCl, 1mM EDTA, pH 7.2) at 4 °C. 100 mM hydrazine monohydrate (63-65
wt%), dissolved in a small volume of reaction buffer with the pH adjusted to 7.2, was slowly added to the protein solution. Reaction was left to nutate for 24 h at 37 °C followed by the addition of 50 mM of hydrazine monohydrate and left to nutate at 37 °C for additional 12 h. Intein cleavage was followed by RP-HPLC and after completion dialyzed against 50 mM Tris, 200 mM NaCl pH 7.2 at 4 °C. The reaction was filtered (0.2 μm membrane filter) and subjected to gravity reverse Ni-NTA affinity purification using binding buffer (50 mM Tris, 200 mM NaCl, 30 mM imidazole pH 7.2). UFM1-hydrazide was collected in the flowthrough, concentrated to 0.75 mM, aliquoted, flash frozen with liquid N₂ and stored until further modification.

2.2 Preparation of symmetrical anhydrides of carboxylic acids

Chloroacetic anhydride 9 (CAS 541-88-8), acetic anhydride 13 (CAS 108-24-7), mono-methyl fumarate 14 (CAS 2756-87-8), glycidol 7 (CAS 556-52-5) and 4-pentynoic acid 15 (CAS 6089-09-4) were purchased from Sigma Aldrich and used without additional purification.

2.2.1 Glycidic acid synthesis

\[
\text{Glycidol 7 (±)} \xrightarrow{\text{RuO}_2 \cdot x \text{H}_2 \text{O, NaIO}_4, \text{CCl}_4: \text{H}_2 \text{O}} \text{Glycidic acid 8}
\]

Glycidic acid 8 was synthesized from (±)-glycidol 7 as reported previously. Briefly, to a (±)-glycidol (13.5 mmol, 1 equiv) in CCl₄:CH₃CN:H₂O (7:7:1) under N₂, ruthenium dioxide dihydrate (2.4 mol%) was added. Reaction mixture was stirred for 5 min followed by addition of NaIO₄ (0.05 mol, 4 equiv.) in portions over 15 minutes. Reaction was stirred overnight at room temperature and stopped by adding silica gel and evaporating to dryness. The solid residue was filtered through short silica column with EtOAc as an eluent. Filtrate was concentrated under reduced pressure to result glycidic acid 8 as a yellow oil with a 65% yield. Without additional purification product was used for generation of an anhydride 10. ¹H NMR (400 MHz, CDCl₃) δ 10.04 (bs, 1H, COOH), δ 3.50 (dd, 1H, CH), δ 3.02 (ddd, 2H, CH₂); ¹³C NMR (400 MHz, CDCl₃) δ 174.8 (COOH), 46.87 (CH), 46.63 (CH₂).
2.2.2 General procedure for synthesis of anhydrides 10, 11, 12

\[
\text{R-OH} \quad \text{DCC} \quad \text{CH}_2\text{Cl}_2, 30 \text{ min, } 0^\circ\text{C} \quad \text{R-O} - \text{O-R}
\]

To a solution of carboxylic acid (0.075 mmol, 1.0 equiv.) in CH\(_2\)Cl\(_2\) at 0°C was added dicyclohexylcarbodiimide (0.037 mmol, 0.49 equiv) and the solution was stirred for 30 min. The precipitate was filtered off and resulting solution was concentrated under reduced pressure to afford symmetrical anhydrides 10, 11, 12. Anhydrides were further used for protein modification without additional purification.

2.3 Procedure for UFM1-hydrazide modification

UFM1 C-terminal hydrazide 1 was buffer exchanged to the reaction buffer (100 mM NaPhos, 50 mM NaCl pH 3.0) and diluted to a final concentration of 150 μM. Symmetrical carboxylic acid anhydrides 9, 10, 11, 12 or 13, were pre-dissolved in 10 μl of DMF and added to the reaction mixture at a final concentration of 30 mM (200 equiv). To check the reaction progress, the reaction was subject to analytical RP-HPLC immediately after the addition of anhydride. RP-HPLC chromatograms and HRMS data are shown in Supplementary Figures 1 – 6. The reaction mixture was dialyzed against 50 mM Tris, 200 mM NaCl pH 7.2 buffer, spin-filtered to reach 750 μM concentration, aliquoted, flash frozen with liquid N\(_2\) and stored until further usage.

2.4 Stability tests for UFM1 probe 2

UFM1 α-chloroacetyl probe 2 was buffer exchanged using size-exclusion columns to PBS buffer and diluted to 150 μM concentration. Directly after buffer exchange an aliquot of the sample was submitted to LC-MS/MS (Waters Xevo TQ-S). Further sample was left to incubate in PBS buffer at room temperature and aliquots were submitted to LC-MS/MS after 30 min, 1h, 2h, 4h, 12h, 24h, 36h, 48h, 72h and 144h (Supplementary Table 7).
2.5 Probes used for \textit{in vitro} crosslinking assays

![Imagery representing probes 2, 3, 4, 5, and 6]

2.5.1 Crosslinking with E1s

UFM1 variants bearing C-terminal electrophilic probes 2 – 6 at 40 μM were incubated with 10 μM of either UBA1, UBA5 or UBA5 C250A, 5 mM MgCl$_2$ with or without addition of 2.5 mM ATP in PBS for 30 minutes at 37 °C. Reactions were quenched by the addition of 2 x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes and subsequently resolved by SDS-PAGE gel electrophoresis. Crosslinking was visualized by Coomassie staining. Gel images are shown in \textit{Supplementary figure 7}.

2.5.2 Crosslinking with E2s

UFM1 variants bearing C-terminal electrophilic probes 2 – 4, 6 at 100 μM were incubated with a panel of E2s: UBE2K, UBE2B, UBE2L3, UBC12, UBC9, UFC1 wt and UFC1 C116A at 15 μM in PBS for 1 h at 37 °C. Reactions were quenched by the addition of 2 x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes and subsequently resolved by SDS-PAGE gel electrophoresis. Crosslinking was visualized by Coomassie staining. Gel images are shown in \textit{Supplementary figure 8, 9}.

2.5.3 Crosslinking with DUBs

UFM1 variants bearing C-terminal electrophilic probes 2 – 6 at 75 μM were incubated with a panel of DUBs: USP21, SENP8, SENP1, YUH1, UFSP2 wt and UFSP2 C302A at 15 μM in PBS for 1 h at 37 °C. Reactions were quenched by the addition of 2 x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes and subsequently resolved by SDS-PAGE gel electrophoresis. Crosslinking was visualized by Coomassie staining. Gel images are shown in \textit{Supplementary figure 10, 11}.

2.5.4 Crosslinking of probe 2 with UFSP2, digestion and MS/MS analysis

UFM1 C-terminal probe 2 at 75 μM was incubated with UFSP2 at 15 μM in PBS for 1 h at 37 °C. 25 mM (NH$_4$)$_2$HCO$_3$ pH 8 was added to the reaction mixture. Samples were reduced by the addition of 10 mM DTT in (NH$_4$)$_2$HCO$_3$ for 45 minutes at 50 °C. Samples were alkylated with
50 mM iodoacetamide for 1 h at room temperature in the dark. Samples were further subjected to trypsin digestion (1:50 ratio) overnight at 37 °C. Samples were desalted using C18 Zip-Tips, concentrated under vacuum to dryness and resuspended in 5 vol% CH$_3$CN with 0.1 vol% formic acid. Cysteine residues were searched with a variable modification for carboxyamidomethylation (+57.02 C), methionine oxidation was searched with a variable modification (+15.99 M), glutamine and asparagine were searched with a variable modification for deamidation (-1.03 NQ). UFSP2 cysteine modification with UFM1 probe was searched with a variable modification for Val-Gly addition (+171.1 VG). Peptides were required to have at least one tryptic terminus and up to two missed cleavage sites.

2.6 Crosslinking of probes in lysates

2.6.1 Labeling of endogenous enzymes in cell lysates
FLAG-UFM1 variants bearing C-terminal probes 2 – 6 at 25 μM were incubated with cell lysates with a total protein amount of 0.3 mg for 2 h at 37 °C. Reactions were quenched by addition of 4 x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes and subject to SDS-PAGE gel electrophoresis. Proteins were transferred onto PVDF membrane and bands were further visualized with Western Blot against FLAG. WB image is shown in Supplementary figure 12.

2.6.2 Labeling of spiked UFSP2 in cell lysates
For all labeling reaction, 10 μM of UFSP2 wt was pre-incubated with cell lysates with total protein amount of 0.3 mg for 30 minutes at 37 °C. 25 μM FLAG-UFM1 variants bearing C-terminal probes 2 – 6 were added and reaction was further incubated for 1 h at 37 °C. Reactions were quenched by addition of 4x SDS-PAGE loading buffer supplemented with 2-β-mercaptoethanol, boiled at 95 °C for 5 minutes and subject to SDS-PAGE gel electrophoresis. Proteins were transferred onto PVDF membrane and bands were further visualized with Western Blot against FLAG. WB image is shown in Supplementary figure 13.

2.7 Knockdown and Knockout Cell Line Generation
HCT116-dCas9-KRAB and HEK293T-dCas9-KRAB (Addgene #102244) cells were transduced with sgRNAs targeting the upstream promoter region (sgUFM1: GAAGAGATGAAGACTGCGTG) for transcriptional knockdown. UFSP2 knockout HEK293T cells were generated using plasmid-based delivery of Cas9 and sgRNAs. Briefly, HEK293T cells were transfected with SpCas9(BB)-2A-GFP (pX458; Addgene #48138) plasmid carrying sgRNAs targeting UFSP2 (sgUFSP2-sgRNA1- AATAAGAGGAGGCTTGATT).
2.8 Electroporation experiments

FLAG-UFM1 variants were delivered into HEK293T-dCas9-KRAB-sgUFM1 cells using a Lonza 4D-Nucleofector according to manufacturer’s protocol with slight modifications. Briefly, 15 M cells were mixed in 120 μl of Lonza SF buffer and 7.5 nmol of each FLAG-UFM1 variant. The mixture was then equally split into two nucleofection cuvettes and nucleofected using pre-programmed HEK293T code on the Lonza 4D-nucleofector. Cells were then transferred into a 15 cm plate for 24 h recovery. The next day, cells were harvested and lysed in 500 μl of RIPA buffer on ice for 10 min. Cells were spun at 20 x g for 5 min at 4 °C to remove cell debris and nuclei. 450 μl of lysates were transferred to a screw cap tube and SDS was added to a final concentration of 2% and boiled at 95 °C for 5 min for denaturation. The denatured lysates were then transferred to a 15 ml falcon tube and 9.5 ml of RIPA buffer was added to dilute the SDS to a final 0.1% before FLAG-immunoprecipitation.

2.9 Immunoprecipitation on anti-FLAG beads

For each FLAG-IP, 100 μl Pierce Anti-DYKDDDDK Magnetic Agarose Beads were pre-equilibrated with RIPA lysis buffer. Cell lysates with nucleofected probes 2 and 6 were incubated with anti-FLAG magnetic beads for 1 h at room temperature. Samples were washed twice with 1 ml of high-salt RIPA lysis buffer, followed by a final wash with 1 ml regular RIPA lysis buffer. The samples were then prepared for on-bead digestion and tandem mass spectrometry analysis. Samples were visualized with Western Blot against UFM1 and against UFSP2.

2.10 On-bead digestion

Beads were washed twice with 100 μl of digestion buffer (10 mM Tris, 2 mM CaCl₂, pH 8.2) and the wash was discarded. The samples were resuspended in 45 μl of 10 mM Tris, 2 mM CaCl₂, pH 8.2 buffer. For reduction and alkylation, 0.9 μl 100 mM TECEP and 1.4 μl 500mM IAA were added and samples were incubated at 60 °C for 30 min. in the dark. For tryptic digestion, 45 μl of 10 mM Tris, 2 mM CaCl₂ buffer (pH 8.2), 2 μl trypsin (300 ng/μl in 10 mM HCl), 0.5 μl of 1 M Tris pH 8.2 (to adjust pH to pH 8) were added. The samples were also subjected to microwave assisted digestion at 60 °C for 30 min. Finally, supernatants were collected, and the peptides were extracted from beads with 150 μl of 0.1% TFA / 50% CH₃CN. The supernatants were dried and resuspended in 20 μl ddH₂O + 0.1% formic acid. Peptide concentration was measured, and samples were 1:100 diluted and transferred to the autosampler vials for Liquid chromatography-mass spectrometry analysis (LCMS/MS). Cysteine residues were searched with a variable modification for carboxyamidomethylation (+57.02 C), methionine oxidation was searched as a variable modification (+15.99 M).
3. SUPPLEMENTARY FIGURES

3.1 Analytical data for UFM1 C-terminal probes

3.1.1 Analytical data for modified UFM1 hydrazide 1

Supplementary Figure 1. Characterization of UFM1 hydrazide 1. a, structure of 1. The product was analyzed by LC-HRMS. b, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. c, HR-MS (ESI-MS), found [M]: 9879.2508 (upper panels), calculated for $C_{444}H_{712}N_{118}O_{134}S$: 9878.2388 (lower panel).
3.1.2 Analytical data for modified UFM1 probe 2

**Supplementary Figure 2.** Characterization of UFM1 C-terminal probe 2. a, structure of 2. The product was analyzed by LC-HRMS. b, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. without prior purification. c, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. after dialysis. d, HR-MS (ESI-MS), found [M]: 9955.2292 (upper panels), calculated for C_{446}H_{713}ClN_{118}O_{135}S: 9955.2112 (lower panel).
3.1.3 Analytical data for modified UFM1 probe 3

**Supplementary Figure 3.** Characterization of UFM1 C-terminal probe 3. a, structure of 3. The product was analyzed by LC-HRMS. b, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. without prior purification. c, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. after dialysis. d, HR-MS (ESI-MS), found [M]: 9991.2483 (upper panels), calculated for $C_{449}H_{716}N_{118}O_{137}S$: 9990.2548 (lower panel).
3.1.4 Analytical data for modified UFM1 probe 4

**Supplementary Figure 4.** Characterization of UFM1 C-terminal probe 4. **a**, structure of 4. The product was analyzed by LC-HRMS. **b**, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. without prior purification. **c**, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. after dialysis. **d**, HR-MS (ESI-MS), found [M]: 9948.2749 (upper panels), calculated for C_{447}H_{714}N_{118}O_{136}S: 9948.2442 (lower panel).
3.1.5 Analytical data for modified UFM1 probe 5

**Supplementary Figure 5.** Characterization of UFM1 C-terminal probe 5. a, structure of 5. The product was analyzed by LC-HRMS. b, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. without prior purification. c, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. after dialysis. d, HR-MS (ESI-MS), found [M]: 9959.2570 (upper panels), calculated for \( \text{C}_{449}\text{H}_{716}\text{N}_{118}\text{O}_{135}\text{S} \): 9958.2650 (lower panel).
3.1.6 Analytical data for modified UFM1 probe 6

Supplementary Figure 6. Characterization of UFM1 C-terminal probe 6. a, structure of 6. The product was analyzed by LC-HRMS. b, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. without prior purification. c, RP-HPLC using a gradient of 20 to 90% acetonitrile over 14 min. after dialysis. d, HR-MS (ESI-MS), found [M]: 9921.2289 (upper panels), calculated for C_{446}H_{714}N_{118}O_{135}S: 9920.2493 (lower panel).
3.2 Stability tests of UFM1 probe 2

**Supplementary Figure 7.** Stability test of the α-acetylchloride probe 2 in PBS. Analysis by ESI-MS, spectra with deconvolution by maximum entropy shown. T₀, UFM1 hydrazide; T₁ = immediately after modification; T₂ = 30 min., T₃ = 1h, T₄ = 2h, T₅ = 4h, T₆ = 12h, T₇ = 24h, T₈ = 36h, T₉ = 48h, T₁₀ = 72h, T₁₁ = 144h incubation.
3.3 SDS-PAGE analysis of UFM1 probes *in vitro* crosslinking with E1s, E2s, DUBs

3.3.1 SDS-PAGE analysis of UFM1 probes *in vitro* crosslinking with UBA1 and UBA5

**Supplementary Figure 8.** Coomassie-stained SDS-PAGE analysis for *in vitro* crosslinking of UFM1 C-terminal probes 2 – 6 with E1s: a, UBA1 (Ub) and b, UBA5 (UFM1). Cropped gel is shown in Figure 4A in the main text.

3.3.2 SDS-PAGE analysis of UFM1 probes *in vitro* crosslinking with a panel of E2s

**Supplementary Figure 9.** Coomassie-stained SDS-PAGE analysis for *in vitro* crosslinking of UFM1 C-terminal probes 2 – 4 with UBE2K, UBE2B, UBE2L3, UBE2D3, UBC12 and UBC9: a, probe 2; b, probe 3; c, probe 4.
3.3.3 SDS-PAGE analysis of UFM1 probes *in vitro* crosslinking with UFC1

*Supplementary Figure 10.* Coomassie-stained SDS-PAGE analysis for *in vitro* crosslinking of UFM1 C-terminal probes 2 – 4 with UFC1 C116A and UFC1 wt. Cropped gel is shown in *Figure 4B* in the main text.
3.3.4 SDS-PAGE analysis of UFM1 probes \textit{in vitro} crosslinking with a panel of DUBs

\textbf{Supplementary Figure 11.} Coomassie-stained SDS-PAGE analysis for \textit{in vitro} crosslinking of UFM1 C-terminal probes 2 – 5 with USP21, SENP8, SENP1 and YUH1: \(a\), probe 2; \(b\), probe 4; \(c\), probe 3; \(d\), probe 5.

3.3.5 SDS-PAGE analysis of UFM1 probes \textit{in vitro} crosslinking with UFSP2
**Supplementary Figure 12.** Coomassie-stained SDS-PAGE analysis for *in vitro* crosslinking of UFM1 C-terminal probes 2 – 5 with UFSP2 C302A and UFSP2 wt. Cropped gel is shown in *Figure 4C* in the main text.

**3.4 Western blots of UFM1 C-terminal probes incubated with lysates**

**3.4.1 Western blots of UFM1 probes 2 – 6 incubated with HCT116 and HCT116 *UFM1* KD whole cell lysates**

**Supplementary Figure 13.** Crosslinking of probes 2 – 6 with endogenous proteins in: a, HCT116 *UFM1* KD whole cell lysates; b, HCT116 whole cell lysates. Visualization with Western Blot against FLAG. Cropped gel a is shown in *Figure 5A* in the main text; c, d Western Blots of the repeated independent experiments for a and b; stain-free visualization used as a loading control.
3.3.2 Western blots of UFM1 probes 2 – 6 incubated with spiked UFSP2 in HEK293T whole cell lysates

Supplementary Figure 14. Crosslinking of probes 2 – 6 with spiked UFSP2 in HEK293T whole cell lysates. Visualization with Western Blot against FLAG.

3.4.3 Western blots of UFM1 probes 2, 6 nucleofected into HEK293T UFM1 KD and HEK293T UFM1 KD, UFSP2 KO cell lines

Supplementary Figure 15. Nucleofection of UFM1 C-terminal probes 1 and 6 into HEK293T UFM1 KD; HEK293T UFM1 KD and UFSP2 KO cell lines, followed by lysis and immunoprecipitation on the anti-FLAG resin. Visualization with Western Blot against UFM1
and against UFSP2. * Indicates anti-mouse secondary antibody IgG background bands. Blots of two independent experiments are shown. Cropped blots are shown in Figures 5B, 5C in the main text.

4. SUPPLEMENTARY TABLES

4.1 Primers, templates, and PCR methods used for molecular cloning.

| Gene variant | Template | Forward primers (5’-3’) | Reverse primers (5’-3’) | PCR |
|--------------|----------|-------------------------|-------------------------|-----|
| UFC1 C116A   | wt       | TGCATTACGGGTGATGCCGGCTG | AACACGATCAGCGGAAATAATACG| Q5  |
| UFSP2 C302A  | wt       | GCTGGGCGCGGCTTATGCATCTCGC | CATTGTCATCTATGCAGCTCTG | Q5  |
| UBA5 C250A   | wt       | GGTGTTCGCGCAGGAGGCTGCC | TTCACGTTTCAGGGGGGGGGGGGATCATATTTG | Q5  |

Supplementary Table 1. Primers and PCR methods used for UFC1 C116A and UFSP2 C302A point mutations.

4.2 Protein sequences

| Protein          | Amino acid sequence                                                                 | Comments                        |
|------------------|-------------------------------------------------------------------------------------|---------------------------------|
| Flag-UFM1-       | MDYKDDDDKSKVFSKILTSDPRLPYSVLSVPESTPTAVLKFAAE FKVPAAATSAITNDGGINPAQTAGNVFLKGSERIIPRDRVCTG DALVALPEGESVRIADIVPGARPNSDAIDLKLVRHGPVLADRLF HSGEHPVYTVRTVEGLVGTVTANHPLLCLVDVAGVPTLLWKLEIEK PGDYAVIQRSAFSVDCAGFARGKPEAPTTTVGVPGLVRFEAHHR DPDAQAIADLTGFRYYAKVASVTDAGVQPVYSLRVETDHAPITNGFVSHALEHHHHH | His6-tagged cleavable intein     |
| GyrA-His<sub>6</sub>-SUMO-UFSP2 | GSSHHHHHHGSDSEVQNEAKPEVKPEVKPETHINLKVSDGSSIEFFK IKTTPSLRRLMEAFAAKRQGKEMDSLRFLYDGIRIADQTPEDLDMEDNDIIEAHREQIGMVISESMDILFIRGGLDAPQLATPNEIFLKLKA LKHVLSDSTKLSSNVALVRICHSIVERYISPSSDNTIPEGLTADASAC KNILRFIQFEPEDIKRKMRKDOKLSDMHQIVNIDLLEMSTSLA | SENP1-cleavable His6-SUMO-tag    |
| Protein | Sequence | Comments |
|---------|----------|----------|
| **His6-** UFC1 | AVTPIERESGHHYVNMTLPVDAVISVAPEETWKVRKLLVDAIHNLQDMEKILYKMT GSTIVVPPELHFLLPGKKNLVTYSPSG1PDDQLOQARVKEHLDFNLPHDRFYKRSNAYHFDPDPYKDGYIRMPHYNLPPNMETGMIVQGIGYHYHHMQRIRDDINGWCGARYSLQTICSWFQHQGTYERSIPTHEIQALVDAKPGATVGSQWSIGIEVQLNQLIGITSKLFVSQGSEIASQGRELHFNQSEGTPVMIWGVvlahtlgVANNEITGG1KFLILDPHYTADELQVILEKGCWKGPDFWKNQDYNLCLPQRPNNI | TEV-cleavable His6-tag |
| **His6-** SUMO-UBA5 | GSSHHHHHSSGAENLYFGADEATRTRVSEIPVTLKTNAQPRDLSELWQRLKEEYQSLRYYVENKNKANDWDWFRLIESNKEGTRWFQGKGYWHDDLKYEFDIEEIPITPTAPAIEAPVLEDGKTAKMYRGKICLTDHFPIQLRANNPFLGHALMLAGLPWLALEVDPDIQKQVIQHKECNQ | SENP1-cleavable His6-SUMO-tag |
| **UBA1-** His10 | MAKNGSEADIEGLYSLQRYLVGHEAMKRLQTSVLSLVSGLRLGVEIAKNIIllGKVLAVTLIDQPOTAQLDSQFYLREEQ1GKRNAESQPRLAELNSYVPVATYGLPLEDFLSGFQVVLTNTPLEDQLRVGEFCHNRGKLWADTRGLQFQLCDFGEMEILTDSNEQPLSAMVSMVTQDNPGVVTCLDEARGFEGDFVIPSFSEVQMELNGNQPEIKVLGYPYTFSCIDTNSFSDYIRGGIVSVQKVPKISFKSLVASLAEPDFVVTDFAKFSRPQALHIGQFALHOFQCAQHGRPPPRNEEAELVALAQAVNARALPAVQQNLDFDLIRKLAYVATGDALIPAFIGLAAQEVMKACSGKFMPIMQWLYFADLCELPLVKEVLTDEDKCLQRQNYDGQVAFSGDLQEKLGQKQKYFLVGAAGICEKNFMAMIGLCGEGGEEIIVTDMTIEKSNLNRQFLRPPWVTLKSDTAAAARQMNPHIRVTSHQNRVGPDETIRYDDDFQNLQDVGALNDVARMYMDRCCVYQKPLLESGTGLGTKGNVQVVFPLTSESYSSQDPPEKSIPICTLKNFPNAIEHTLQWARDE |
| Protein | Sequence |
|---------|----------|
| **His6-**<br>**YUH1** | GSSHHHHHHGSGAENLYFQGGSMSGENRAVPIESNPVEFNTNAHK<br>LGKNEWAYFYISLTERPELLAFLPVTKAIYVLFPINEDRKSSTSQQ<br>QQSSSYDCNFQSKVQNAUYALHSLSNQSLLEPSGDILNQLS<br>QSDTSSKNRFDVTDQFVLSVIKENRVTSTQSEAPATATNLLHYITYVEENGIFELDRNLGSPLYLGKSDPATEGDLEQELVRVRA<br>SYMENANEDVLNFMGLGPNEE |
| | **TEV-cleaveable**<br>**His6-tag** |
| **His6-**<br>**SUMO-**<br>**USP21** | GSSHHHHHHGSGAENLYFQGGSMSGENRAVPIESNPVEFNTNAHK<br>LGKNEWAYFYISLTERPELLAFLPVTKAIYVLFPINEDRKSSTSQQ<br>QQSSSYDCNFQSKVQNAUYALHSLSNQSLLEPSGDILNQLS<br>QSDTSSKNRFDVTDQFVLSVIKENRVTSTQSEAPATATNLLHYITYVEENGIFELDRNLGSPLYLGKSDPATEGDLEQELVRVRA<br>SYMENANEDVLNFMGLGPNEE |
| | **SENP1-cleaveable**<br>**His6-**<br>**SUMO-tag** |
| **His6-**<br>**SENPI** | GSSHHHHHHGSGAENLYFQGGSMSGEPTIMEEMEKEIKNVRFNGNQDEVLE<br>SEAFLRTITRDQITLNLHLWNEIDINFNYMNMERSKELGKLPSVH<br>AFNTFFFTKLATQYAGVKRWTKQVDVFSVDILLVLGIVHCLAV<br>VDFRKKNTITYDSMGMGINNEACRLLQILKQESIDKKRKEFDTNGQ<br>LFSKKSQIEIPQQMGSDAGMFACKYADCITKDRPINFTQQHMPYFRK<br>RMVWEILHRL |
| | **Thrombin-cleaveable**<br>**SENPI** |
| **His6-**<br>**SENPS8** | GSSHHHHHHGSGAENLYFQGGSMDPVVSLYMSLSSLQRSDVSLDDPSWLN<br>NDHIIGAFEFYFANSQFHDSDHSVFSIFPQPIKCTSNPAEIAMF<br>LEPLDLPNKRVFLAINDNSNQAAGGLHSSLLVLQDKNSFFHYDSH |
| | **Thrombin-cleaveable**<br>**SENPS8** |
### Supplementary Table 2

Amino acid sequences with tags of UFM1, UFC1, UFSP2, UBA5, UBA1, YUH1, USP21, SENP1, SENP8, UBE2K, UBE2B, UBE2L3, UBC12 and UBC9 used in this study.

| Tag        | Sequence                                                                 | TEV-cleavable | His6-tag |
|------------|--------------------------------------------------------------------------|---------------|----------|
| His6-UBE2K | SRSNSVHAKQVAEKLAEFLGRKDGKLAFVEEKAPQAQQNSYDAGMYVICNTEALCNQRFPQTESTLLQLLTPAYITKGGWKLIGLIALATLAK* |               |          |
| His6-UBE2B | MGSSHHHHHHHHSSGAENLYFQGMANIAVQRKREKVEVLKESETSNQIKVDLVDNENFTELGEREAGPPDTMPGREGYQLEIKIPETYPFNPKPVRFITKISHNPISSSVACTILDGQWAAMTLRVTLLLQALLAEEPDPPQDAVVANQYKQPENPKQTARLWAHVVAGAPVSSPEYTKKIE-NLSAMGFDRNAVAILSSKSWDVETATEELLLSN |               |          |
| His6-UBE2L3| MGSSHHHHHHHHSSGAENLYFQMSTPARRRLMRDFKRLQEDDPGVGSGAPSENNIM9WNAVIQFPEPTFEDGFKLVIEFSEEYPKPKPTVRLSKMFHPNYADGSICLDIQLNWR6PSTVDVSSILTSIQSLLDEPNPNSPANSQAAQLYQENKREYERKRVSAIVEQSWNDS |               |          |
| His6-UBC12 | MGSSHHHHHHHHSSGAENLYFQMIKLFSLKKQKKEESEAGTTGKSSKASAAQLIRIQKDINENLPKTCDISDFSDDDPDLNFKLVICPDEFYSGKFVFSFKVGQYPHDPPKVCKETMVHPNIDLEGVNCDNVLINREDWKPVLTINSIIYGLQYLFLEEPNPEDPLNKEAEEVLOQNRRLEFQNVQRSMGQGIGSTYFERCLK |               |          |
| His6-UBC9 | MGSSHHHHHHHHSSGAENLYFQMSGIALSRLAQERKAWRKDHPFGFVAVPTKNPDGMTMNLMWCEAIPEGKGTWEGGFLKRLMKFDYPSSPPKCKFEPPFLFPNVSPTVCLSILEEEDKWRPAITIKQIJQELLNPNIQDPAQAEEAYIQYCNREYERKVRQAQAKKFaps |               |          |

5. SUPPLEMENTARY REFERENCES

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