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Supporting information

Chemical Protein Synthesis Combined with Protein Cell Delivery Reveals New Insights on the Maturation Process of SUMO2

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1. General reagents

Solid Phase Peptide Synthesis (SPPS) was carried out either manually in Teflon filter fitted syringes purchased from Torviq or by using an automated peptide synthesizer (CS336X, CSBIO). 2-chlorotrityl chloride (2-CTC) resin was purchased from Chem-Impex. All protected amino acids were purchased from Chem-Impex and Iris-Biotech. The activating reagents [(6chlorobenzotriazol1-yl)oxy(dimethylamino)methylidene]- dimethyl-azanium hexafluorophosphate (HCTU), 1- [Bis(dimethylamino)methylene]-1H-1,2,3- triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), and 1- Hydroxybenzotriazolemonohydrate (HOBt) were purchased from Luxembourg Bio Technologies. All solvents including *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (ACN), *N*,*N*-diisopropylethylamine (DIEA), Piperidine, Diethyl ether (Et_2O) and Trifluoroacetic acid (TFA) were purchased from Bio-Lab. Triisopropyl silane (TIPS) was purchased from Sigma-Aldrich. 5-Carboxytetramethylrhodamine (TAMRA) was purchased from Aaron Chemicals, Fluorescein isothiocyanate (FITC) was purchased from Chem-Impex. {2[2-(Fmoc amino)ethoxy]ethoxy}acetic acid (PEG) was purchased from apollo scientific. Paraformaldehyde, 4% in PBS was purchased from Affymetrix. 4-20% MOPS gel (MP42G12) was purchased from Merck. Additional miscellaneous chemicals were purchased from Merck, Strem chemicals, and Alfa Aesar.

Caution: TFA, DMF, DCM, ACN, Et_2O, and coupling reagents (HCTU, HATU, and HOBt) are hazardous chemicals with high toxicity and potential health risks. All procedures involving these reagents were conducted in a well-ventilated fume hood, with appropriate personal protective equipment (PPE) including lab coats, chemical-resistant gloves, and safety goggles.

2. List of the protected amino acids used in peptide synthesis

Fmoc-Ala-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Gly-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc- Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)- OH, Fmoc-Cys(Trt)-OH, Fmoc-Nle-OH, Fmoc-Val-OH, Fmoc-Asp(O^{t}Bu)-Thr(ψMe,MePro)-OH, Fmoc-(Dmb)Gly-OH, Fmoc-Leu-Ser(ψMe,MePro)-OH, Fmoc-D-Ala-OH.

3. List of cell culture reagents

Dulbecco's modified Eagle's medium-low glucose (DMEM-LG), fetal bovine serum (FBS), trypsin-EDTA, antibiotics (penicillin/streptomycin), Dulbecco's phosphate-buffered saline (PBS), poly-L-lysine hydrobromide - were purchased from Sigma. Phenol-free Dulbecco's modified eagle's medium (Opti-DMEM), and 10% Pluronic F-68 Non-ionic Surfactant - were purchased from Gibco. Hoechst 33342 solution (20 mM) was purchased from Thermo-fisher. 8 wells µ-Slide for cell culture for confocal microscopy were purchased from ibidi.

4. Antibodies for western-blotting

Rabbit anti-SUMO2 (PA5-27636, invitrogen), rabbit anti-beta actin (Abcam, ab8227), secondary goat anti-rabbit IgG H&L-(HRP) (Abcam, ab6721).

5. Fmoc-SPPS general procedure on 2-CTC resin

2-Chlorotrityl chloride (CTC) resin (0.30-0.80 mmol/g) was initially swelled in a 1:1 mixture of DMF and DCM for 30 minutes, followed by washing with DCM (5 × 10 mL). The resin was then loaded with 0.4 eq. of Fmoc-X-OH (where X represents Gly, Tyr, or Ala) and 2 eq. of DIEA in dry DCM and the reaction was left on a plate shaker for 1 hour at room temperature. After loading, the Fmoc-X-resin was sequentially washed with DCM (5 × 10 mL), DMF (5 × 10 mL), and DCM (5 × 10 mL). Subsequently, it was treated with MeOH (3 × 5 mL) for 3 minutes each to cap any unreacted chlorides, followed by thoroughly washed with DMF and DCM, and dried in a vacuum. The final loading was estimated to be approximately 0.3 mmol/g by measuring the absorbance of the Fmoc group after treatment with DBU.[1] Following the initial amino acid loading, Fmoc-SPPS was performed on an automated peptide synthesizer (CS336X, CSBIO) using 4 eq. of the amino acid, 4 eq. of HCTU, and 8 eq. of DIEA. Two pseudoproline dipeptides, Fmoc-Asp($O^{t}Bu$)-Thr($\psi^{Me,Me}$Pro)-OH and Fmoc-Leu-Ser($\psi^{Me,Me}$Pro)-OH, and two Fmoc-(Dmb)Gly-OH were manually coupled at indicated positions (highlighted in the sequence) using 2.5 eq. of each dipeptide, 2.5 eq. of HATU, and 5 eq. of DIEA, with constant shaking for 1.5 hours. The linker [2-[2-(Fmocamino) ethoxy]ethoxy]acetic acid (PEG) was manually coupled using 4 eq. of amino acid, 4 eq. of HATU, and 8 eq. of DIEA for 1.5 hours. TAMRA was coupled using 1.5 eq. and 1.5 eq. of

HATU, and 3 eq. of DIEA, and shaken for 2 hours. FITC was coupled using 4 eq. and 4 eq. of DIEA for 2 hours. To cleave the peptides from the solid support, the resin was washed five times each with DMF and DCM, then dried under a high vacuum. A cleavage cocktail of TFA, PhOH, H_2O, PhSMe, and EDT in the ratio 82.5:5:5:5:2.5 was added to the resin, and the mixture was agitated for 3 hours at room temperature. The resin was then filtered, and the filtrate was added dropwise to a 10-fold volume of ice-cold diethyl ether and centrifuged at 4500 rpm. The resulting precipitate was purged with nitrogen, dissolved in an acetonitrile-water mixture, and freeze-dried in a lyophilizer to obtain the crude peptide

6. HPLC for peptide analysis and purification

Analytical high-performance liquid chromatography (HPLC) was performed on a Thermo instrument (Dionex Ultimate 3000) using Xbridge® (4.6 × 150 mm, 3.5 µm, 300 Å, BEH C4, waters) analytical column with 1.2 mL/minutes flow rate. Semi-preparative HPLC was carried out on a Thermo instrument (dionex ultimate 3000) using Xbridge® (10 × 250 mm, 5 µm, 300 Å, BEH C4, waters) semi-preparative column with 4.0 mL/minutes flow rate, and preparative HPLC was performed on a Thermo instrument (dionex ultimate 3000) using Xbridge® (19 × 250 mm, 10 µm, 300 Å, BEH C4, waters) preparative column with 15.0 mL/minutes flow rate. All synthetic peptides were purified by HPLC and characterized by mass spectrometry using the LCQ Fleet Ion Trap (thermo Scientific). Calculated masses have been reported as an average isotope composition.
Buffer A: 0.05% TFA in water; buffer B: 0.05% TFA in acetonitrile were used as HPLC mobile phases.

7. General cell culture procedure

U2OS (HTB-96™ ATCC®S) cells were cultured in DMEM-low glucose supplemented with 10% FBS, and antibiotics (penicillin/streptomycin) in a humidified 37 °C incubator at 5% CO_2. To detach cells from culture flasks, the media aspired, and the flask was washed with sterile calcium and magnesium free (W/O Ca and Mg) PBS, after, cells were treated with 0.25% Trypsin + 0.02% EDTA solution and returned to the incubation chamber for 5 minutes. Trypsin was quenched by adding the FBS supplemented media.

For the confocal microscopy imaging, the cell suspension was collected and pelleted (5 minutes at 3,500 x g). Media was then aspirated, and the cell pellet was re-suspended in fresh media. The cell density was determined using an automated cells counter (Countess II, Invitrogen). Then the cells were seeded on poly-L-lysine (PLL) treated 8 well chamber slides (Ibidi) in 3.2×10^4 cells per well and seeded to reach ~90% confluence (24 hours).

8. General procedure for protein loading via multiplexed bead loading (MBL)[2]

Cells were cultured in 8 well ibdi chamber slides to ~90% confluence for microscopy imaging and in 60 mm plates to ~90% confluence for gel analyses. The protein constructs were diluted from DMSO stocks into sterile PBS with 0.1% Pluronic F-68.

All SUMO2 analogues, after HPLC purification, were dissolved in DMSO at a concentration of 8 mM. Refolding was performed by diluting 5 µL from DMSO stock in 1 mL of sterile PBS buffer followed by incubation at 37 °C. After a brief vortex, the solution was centrifuged to separate the insoluble and aggregated proteins from the supernatant containing the soluble proteins. The protein concentration was determined by Bradford assay against a BSA calibration curve, and the concentrations were adjusted to 2 µM final concentration per protein for confocal microscopy and 5 µM for gel analyses. For MBL, equal volumes of each protein were mixed before cell treatment. For loading, the culture medium was aspired, and cells were washed once with warm PBS+0.1% Pluronic F-68 solution. To the washed cells, 150 µL of the protein solution was added (to cover the cells) for confocal microscopy, and 1 mL of protein solution was added for gel analyses. In addition to the protein solution- a monolayer of glass beads (<106 µm, Sigma Aldrich) was sprinkled over the cells (for gel analyses 0.5 mL of glass beads added). The culture slide was tapped on the bench seven times, gently swirling the slide for a total of two times in between tapping and incubated for 3 minutes at 37 °C under 5% CO_2. Following incubation, the cells were washed twice with PBS+0.1% Pluronic F-68 solution to remove the glass beads followed by incubation at 37 °C under 5% CO_2 for different times (depending on the experiment).

9. Fluorescence gel analyses and western blot

After protein loading via MBL, the cells were lysed. First, the cells were detached and pelleted as described previously. Then, the cell pellet was washed twice with cold PBS W/O Ca and Mg, and 50-200 µL of lysis buffer[3] (2X Laemmli sample buffer- 5% (wt/vol) SDS, 25% (vol/vol) glycerol, 150 mM Tris-HCl pH 6.8, 0.01% (wt/vol) bromophenol blue) was added, followed by incubation of 10 minutes at 95 °C then sonicate for 30 seconds. The lysis concentration was determined by Bradford assay against a BSA calibration curve, then, 0.7 M of beta-mercaptoethanol was added to each sample followed by centrifuge at 13,000 x g for 10 minutes at room temperature. The supernatant was collected, and the samples were stored at -20 °C.

Before the gel run, reducing sample buffer X6 was added to the lysate samples followed by incubation at 95 °C for 5 minutes. The samples were run on 4-20% SDS-PAGE. The gel was imaged using Fusion FX6 ECL and a fluorescence detection system.

Then, the gel was electroblotted onto PVDF membranes followed by blocking in InstantBlock solution (Tivan-Biotech) for 10 minutes at room temperature. The membranes were incubated with primary antibodies (as mentioned above) for either 2 hours at room temperature or overnight at 4 °C, followed by 1 hour of incubation with corresponding secondary antibodies. Following washing with TBST, the membranes were incubated with immobilon Crescendo western HRP substrate (Merck Millipore), and the signals were captured using the ECL detection system.

For the experiment presented in Figure 4, the cells were treated 4 hours before protein delivery with 10 µM of proteasome inhibitor MG132 (Sigma). The reason for treating the cells with MG132 relay on a previous study showed that SUMO2/3 conjugates accumulated in response to proteasome inhibitors[4].

10. General method for imaging using confocal microscopy

After the general procedure for protein loading via MBL the culture medium was aspired and washed once with opti-DMEM-low glucose supplemented with 10% FBS, 0.2 mM L-Gln, and antibiotics (penicillin/streptomycin). Then, an Opti medium containing 10 µM of Hoechst solution was added, and the cells were incubated for 10 minutes before imaging.

For the incorporation studies in PML-NBs, after Hoechst staining the cells were washed twice with PBS and then fixed with 4% paraformaldehyde in PBS for 15 minutes in the dark at room temperature, then washed twice with PBS and kept with PBS at 4 °C until imaging.

The distribution of fluorescent proteins in live cells was analysed using a laser scanning confocal microscopy (LSCM) (Confocal Zeiss LSM 710, Axio observer) equipped with C-Apochromate X40/NA1.2 water immersion objective for live cell imaging. All channels were adjusted to 1 AU pinhole settings. Three lasers were used for the different tags; UV laser (Hoechst) – 405 nm (10 mW), Argon multi-line laser (FITC) – 454, 488 and 514 nm (30 mW), Green laser (TAMRA) – 543 nm (10 mW). During LSCM analyses the samples were kept at room temperature. Single representative images were analysed using ZEISS ZEN 3.7 software.

11. Expression and purification of SENP2 active domain and procedure for the cleavage reaction *in vitro*

SENP2 active domain plasmid (addgene # 196355) was transformed to Rosetta™ 2(DE3) Singles™ competent cells (Merck). Then 60 mL LB with selection antibiotics were inoculated with a single colony and were allowed to grow overnight at 37 °C. 10 mL of the fresh cultured were used to inoculate 1 L of LB media, the culture was grown at 37 °C till it reached OD 0.8 and was induced with IPTG at a final concentration of 0.2 mM. The culture was incubated for 18 hours at 16 °C. Upon completion of the protein expression induction by IPTG, cells were precipitated by centrifugation at 13000 x g for 30 minutes. The pellet was resuspended with 15 mL lysis buffer (50 mM Tris, 150 mM NaCl, 20 mM imidazole, pH 8) containing 100 ppm PMSF. Cells were broken by sonication (7 minutes, 40% amplitude, 10 seconds on, 30 seconds off). The lysate was centrifuged (13000 x g for 30 minutes), and the supernatant was collected and completed with lysis buffer up to 25 mL before filtration (0.22 µm). The expressed protein was purified using HisTrap HP column (Cytiva) and then was dialyzed against 50 mM Tris pH 8, 300 mM NaCl, yielding in 35 mg of purified protein per 1 L culture.

SUMO cleavage assay was performed by incubating SENP2 active domain (40 nM final concentration) with 5 µM of SUMO2 analog in assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM DTT). The mixture was incubated at 37 °C, and aliquots were taken in different time points.

The reaction mixture was analysed by analytical HPLC using a C4 column with a gradient of 25-45% buffer B over 20 minutes and ESI-MS for cleavage detection.

12. Sequence alignment of SUMO orthologs and paralogues across different species

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Saccharomyces cerevisiae(Smt3)    MSDSEVNQEAKPEVKPEVKPETHINLKVS-DGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRF 65
H. sapiens (SUMO1)                MSDQEAKPSTEDLG--DKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMNSLRF 64
H. sapiens (SUMO2)                MADEKPKEGVK------TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60
Pantherophis guttatus (SUMO2)     MADEKPKEGVK------TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60
Phascolarctos cinereus(SUMO2)     MADEKPKEGVK------TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60
Vombatus ursinus                  MADEKPKEGVK------TENNDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60
H. sapiens (SUMO3)                MSEEKPKEGVK------TE-NDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMRQIRF 60

Saccharomyces cerevisiae(Smt3)    LYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG-ATY-------- 101
H. sapiens (SUMO1)                LFEGQRIADNHTPKELGMEEEDVIEVYQEQTGG-HSTV------- 101
H. sapiens (SUMO2)                RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-VY--------- 95
Pantherophis guttatus (SUMO2)     RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-MN--------- 95
Phascolarctos cinereus(SUMO2)     RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-AY--------- 95
Vombatus ursinus                  RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-AY--------- 95
H. sapiens (SUMO3)                RFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG-VPESSLAGHSF 103
```

13. Synthesis of SUMO2 (1)

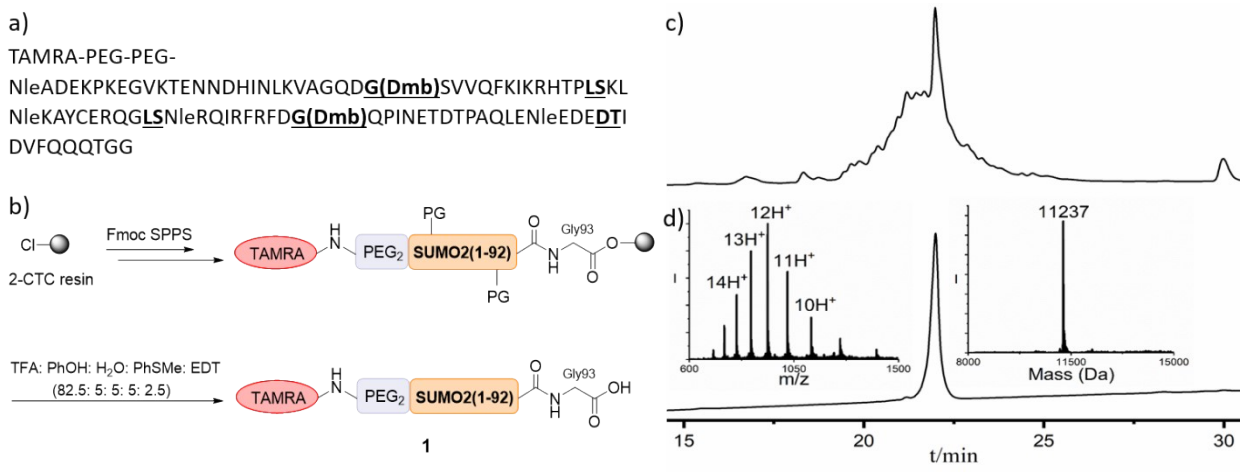


Figure S1. Synthesis of SUMO2 (**1**): a) Sequence of **1**. (b) Schematic representation of synthetic route for **1**. (c) Analytical HPLC chromatogram of crude **1** (detection at 214 nm). (d) Analytical

HPLC and mass analysis of purified **1** with the observed mass 11237.4 ± 0.3 Da, calcd. 11238.6 Da (average isotopes).

SUMO2 (**1**) was synthesized on 2-chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Gly, following described general procedure of Fmoc-SPPS. The crude peptide **1** was purified by preparative HPLC using a C4 column with a gradient of 15-50% B over 50 minutes, yielding peptide **1** with an isolated yield of ~9.4%.

14. Synthesis of SUMO2-VY (2)

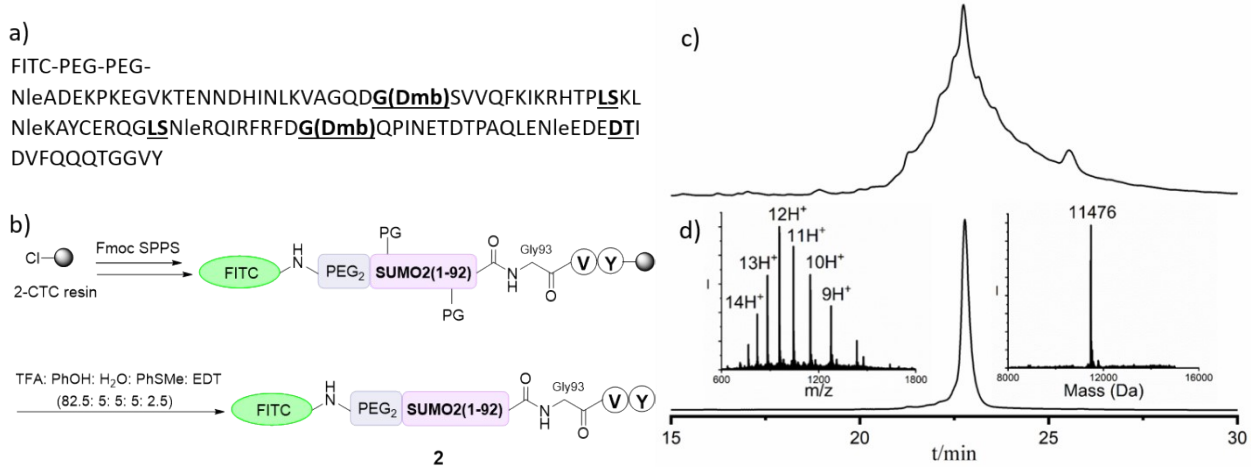


Figure S2. Synthesis of SUMO2-VY (**2**): a) Sequence of **2**. (b) Schematic representation of synthetic route for **2**. (c) Analytical HPLC chromatogram of crude **2** (detection at 214 nm). (d) Analytical HPLC and mass analysis of purified **2** with the observed mass 11476.5 ± 0.5 Da, calcd. 11478.8 Da (average isotopes).

SUMO2-VY (**2**) was synthesized on 2-chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr, following described general procedure of Fmoc-SPPS. The crude peptide **2** was purified by preparative HPLC using a C4 column with a gradient of 25-50% B over 30 minutes, yielding peptide **2** with an isolated yield of ~3.4%.

15. Synthesis of SUMO2-VY (3)

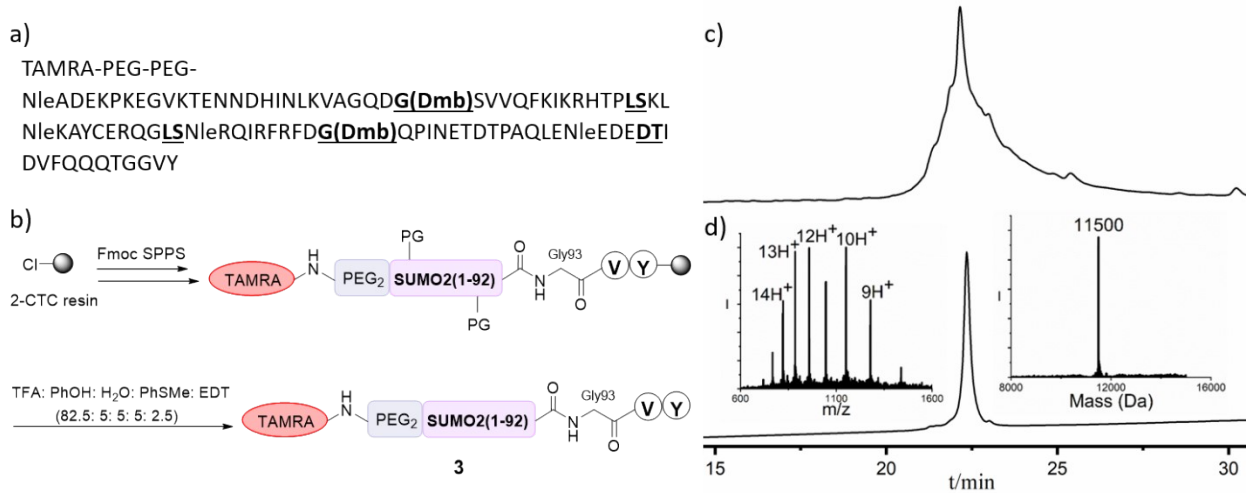


Figure S3. Synthesis of SUMO2-VY (**3**): a) Sequence of **3**. (b) Schematic representation of synthetic route for **3**. (c) Analytical HPLC chromatogram of crude **3** (detection at 214 nm). (d) Analytical HPLC and mass analysis of purified **3** with the observed mass 11499.9 ± 0.5 Da, calcd. 11500.9 Da (average isotopes).

SUMO2-VY (**3**) was synthesized on 2-chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr, following described general procedure of Fmoc-SPPS. The crude peptide **3** was purified by preparative HPLC using a C4 column with a gradient of 25-50% buffer B over 30 minutes, yielding the corresponding peptide **3** with an isolated yield of ~9.2%.

16. Synthesis of SUMO2G93A-VY (4)

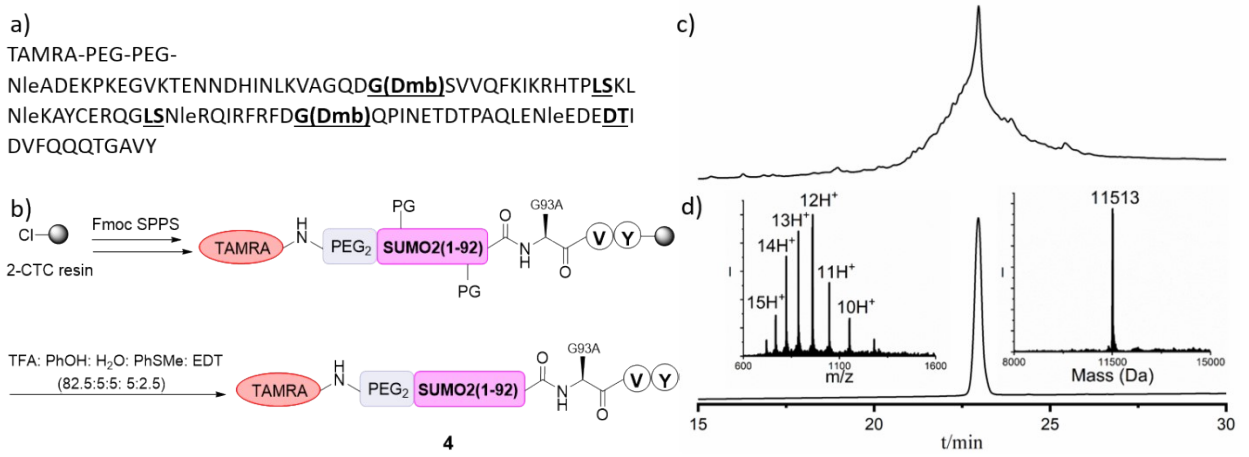


Figure S4. Synthesis of SUMO2G93A-VY (**4**): a) Sequence of **4**. (b) Schematic representation of synthetic route for **4**. (c) Analytical HPLC chromatogram of crude **4** (detection at 214 nm). (d) Analytical HPLC and mass analysis of purified **4** with the observed mass 11512.6 ± 0.5 Da, calcd. 11514.9 Da (average isotopes).

SUMO2G93A-VY (**4**), was synthesized on 2-Chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr, as described in the general procedure of Fmoc-SPPS. The crude peptide **4** was purified by preparative HPLC using a C4 column with a gradient of 15-50% B over 50 minutes, yielding the corresponding peptide **4** with an isolated yield of ~6.5%.

17. Synthesis of SUMO2ΔG93 (5)

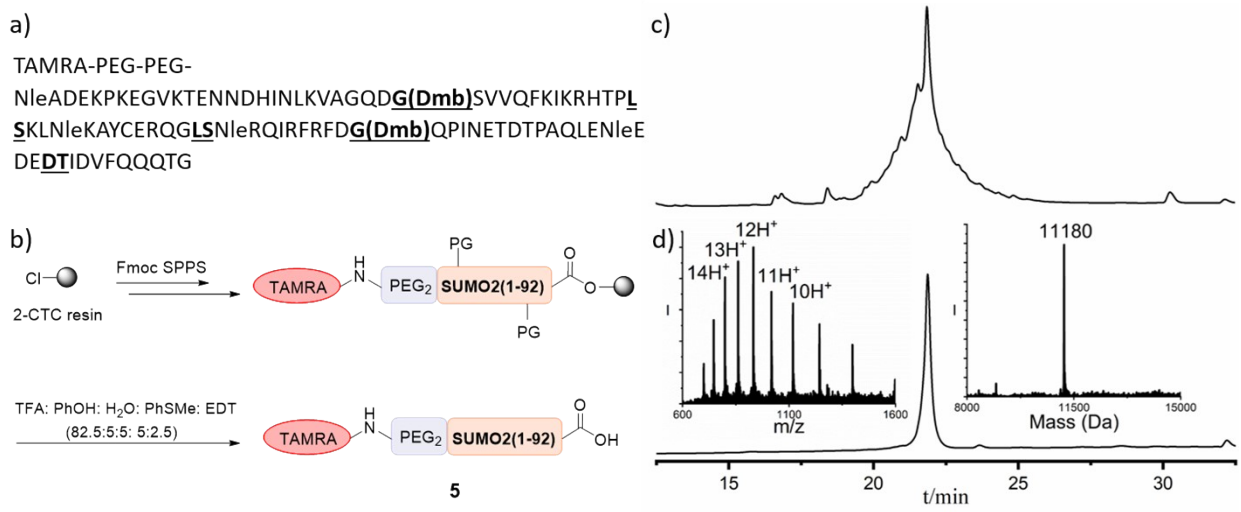


Figure S5. Synthesis of SUMO2ΔG93 (**5**): a) Sequence of **5**. (b) Schematic representation of synthetic route for **5**. (c) Analytical HPLC chromatogram of crude **5** (detection at 214 nm). (d) Analytical HPLC and mass analysis of purified **5** with the observed mass 11179.9 ± 0.5 Da, calcd. 11181.5 Da (average isotopes).

SUMO2ΔG93 (**5**) was synthesized on 2-chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Gly, following described general procedure of Fmoc-SPPS. The crude peptide **5** was purified by preparative HPLC using a C4 column with a gradient of 25-50% buffer B over 30 minutes, yielding peptide **5** with an isolated yield of ~5.7%.

18. Synthesis of SUMO2G93D-Ala-VY (6)

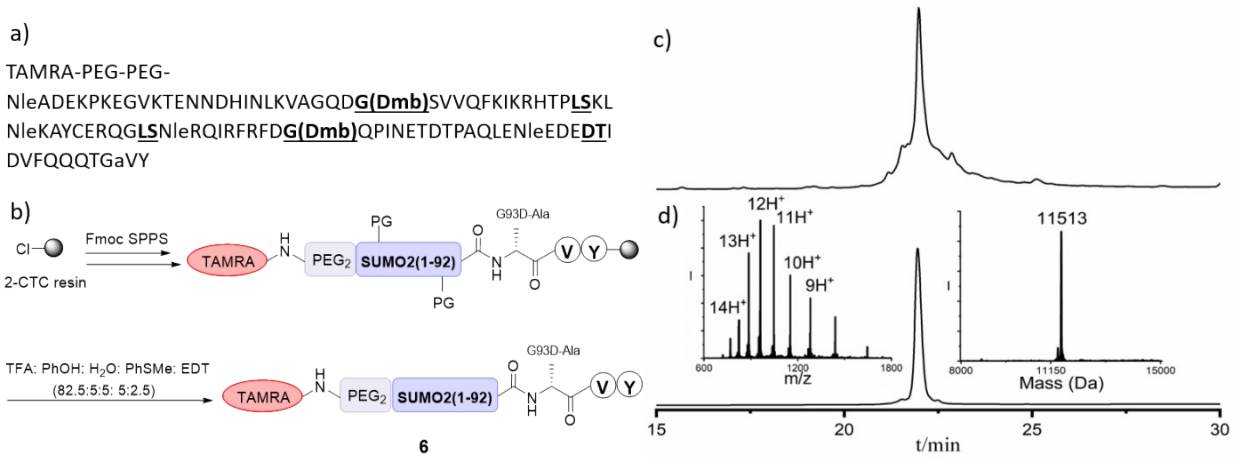


Figure S6. Synthesis of SUMO2G93D-Ala-VY (**6**): a) Sequence of **6**. (b) Schematic representation of synthetic route for **6**. (c) Analytical HPLC chromatogram of crude **6** (detection at 214 nm). (d) Analytical HPLC and mass analysis of purified **6** with the observed mass 11512.8 ± 0.5 Da, calcd. 11514.9 Da (average isotopes).

SUMO2G93D-Ala-VY (**6**), was synthesized on 2-Chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr, following described general procedure of Fmoc-SPPS. The crude peptide **6** was purified by preparative HPLC using a C4 column with a gradient of 25-50% buffer B over 30 minutes, yielding peptide **6** with an isolated yield of ~8.8%.

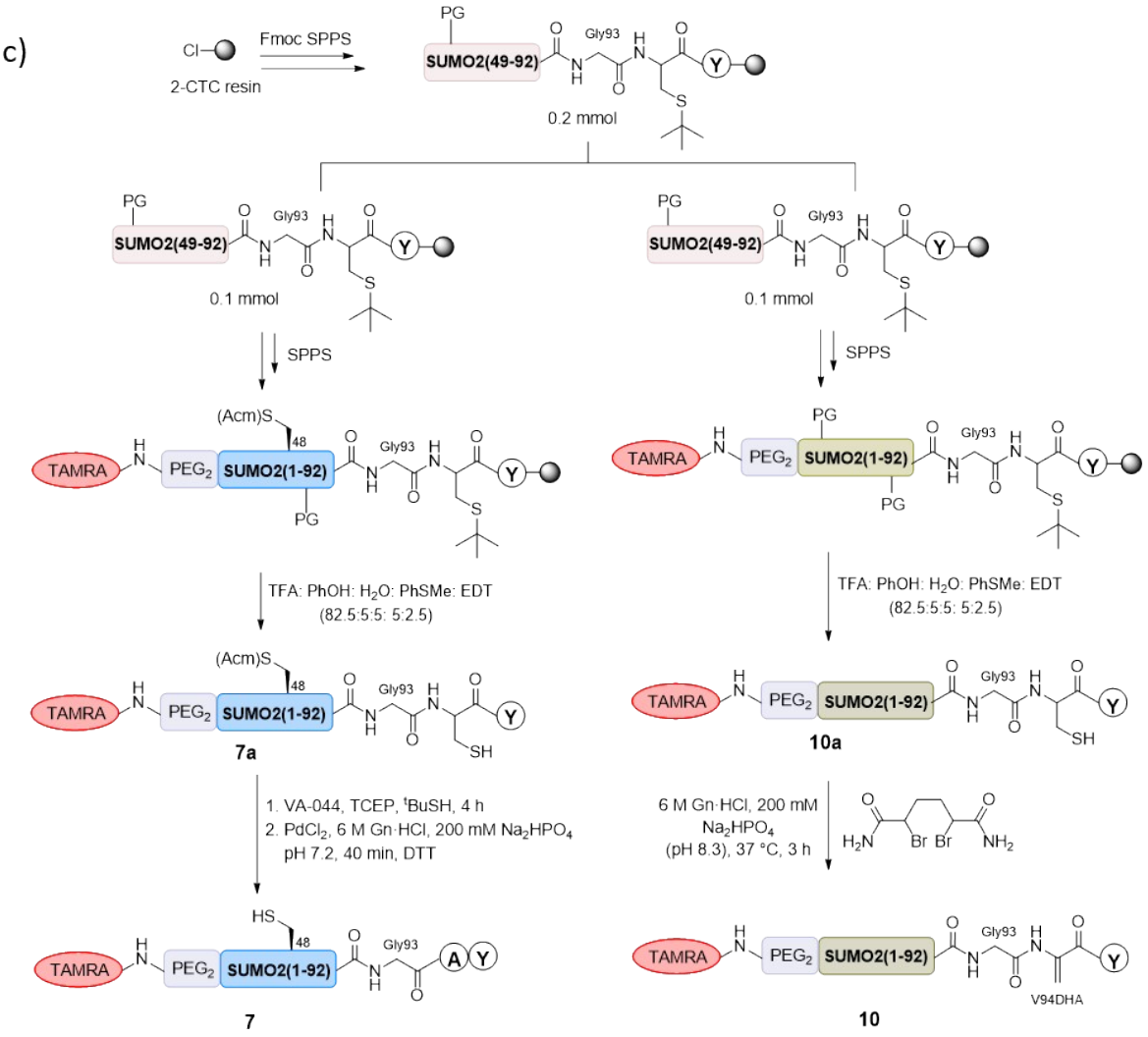
19. Synthesis of SUMO2-AY (7) and SUMO2-EA (10)

To synthesize SUMO2 analogues **7** and **10**, 2-Chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr was subjected to coupling with Cys instead of native Val. The Fmoc-SPPS was continued up to the 49th amino acid (glutamic acid) following the general procedure described. Subsequently, the resin was divided into two equal fractions of 0.1 mmol each, and SPPS was performed separately for further amino acid residues and subsequent reactions as described below.

a) TAMRA-PEG-PEG-NleADEKPKEGVKTENNDHINLKVAGQD**G(Dmb)**SVVQFKIKRHTP**LS**KLNleKAYCERQG**LS**NleRQIRFRF D**G(Dmb)**QPINETDTPAQLENleEDE**DT**IDVFQQQTGGAY

b) TAMRA-PEG-PEG-NleADEKPKEGVKTENNDHINLKVAGQD**G(Dmb)**SVVQFKIKRHTP**LS**KLNleKAY**S**ERQG**LS**NleRQIRFRF D**G(Dmb)**QPINETDTPAQLENleEDE**DT**IDVFQQQTGG(Dha)Y

c)



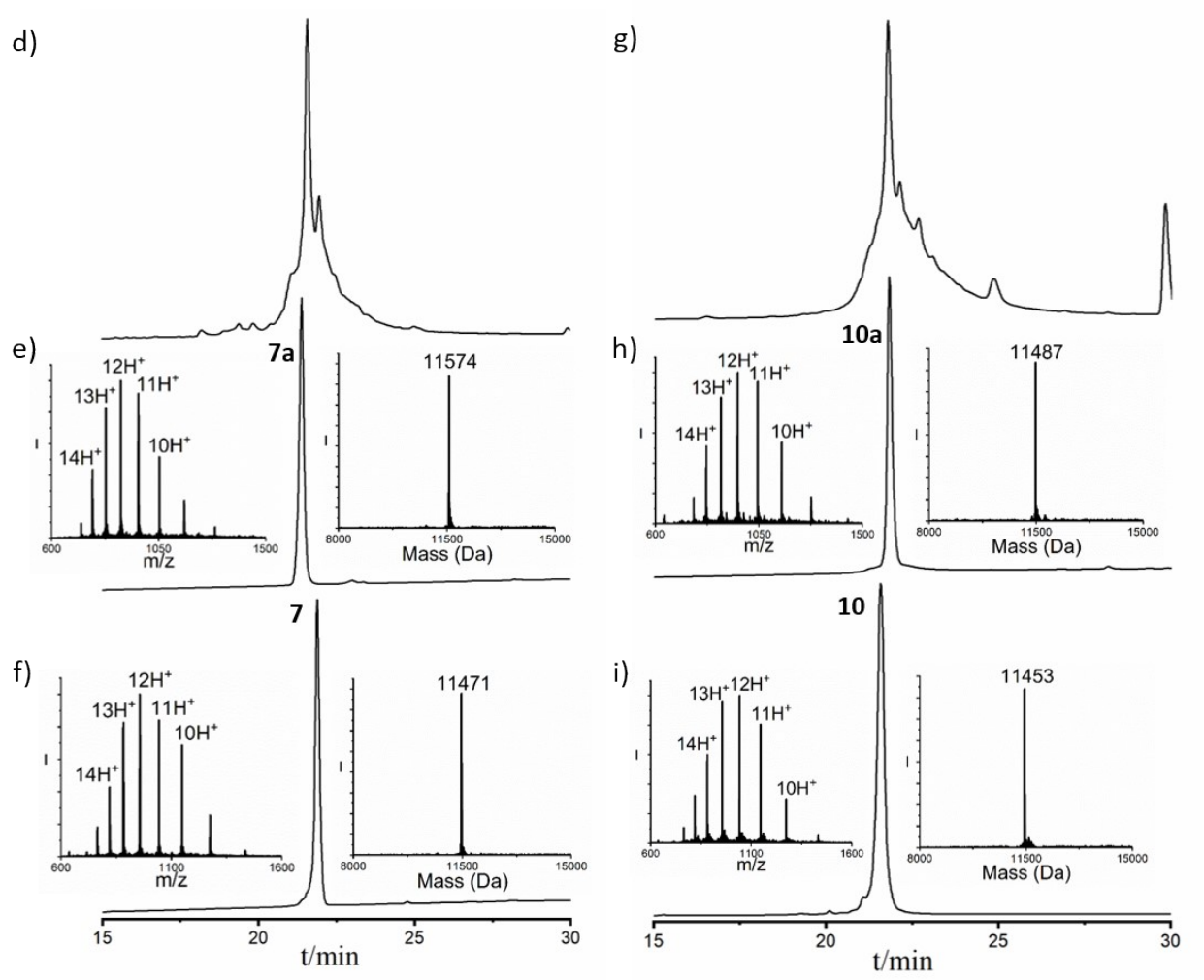


Figure S7. Synthesis of SUMO2-AY (**7**) and SUMO2-V94DhaY (**10**): a) and b) Sequence of **7** and **10**, respectively. c) Schematic representation of synthetic route for **7** and **10**. d) Analytical HPLC chromatogram of crude **7a** (detection at 214 nm). e) Analytical HPLC and mass analysis of purified **7a** with the observed mass 11573.6 ± 0.3 Da, calcd. 11576.0 Da (average isotopes). f) Analytical HPLC and mass analysis of purified **7** with the observed mass 11471.0 ± 0.4 Da, calcd. 11472.8 Da (average isotopes). g) Analytical HPLC chromatogram of crude **10a** (detection at 214 nm). h) Analytical HPLC and mass analysis of purified **10a** with the observed mass 11486.9 ± 0.6 Da, calcd. 11488.8 Da (average isotopes). i) Analytical HPLC and mass analysis of purified **10** with the observed mass 11453.0 ± 0.7 Da, calcd. 11454.7 Da (average isotopes).

Synthesis of SUMO2-AY (7)

Using one fraction of 0.10 mmol from the previously split resin, Cys(Acm) was coupled at the 48th position, and the synthesis of the remaining residues (Figure S7a) proceeded according to the

general procedure of Fmoc-SPPS. The crude peptide was purified by preparative HPLC using a C4 column with a gradient of 15-50% B over 50 minutes, yielding peptide **7a** with an isolated yield of ~7.2%.

Desulfurization: 10 mg (8.64×10^{-4} mmol) of Purified peptide **7a** was dissolved in 6M Gn·HCl, 200 mM phosphate buffer (pH 6) (final concentration 2 mM), was treated with TCEP (30 mg, 0.25 mM), VA-044 (12.0 mg, 50 equiv) and t-BuSH (43 µL) at 37 °C for 4 h. The progress of the reaction was monitored using analytical HPLC (C4 column) with a gradient of 0-60% B over 30 minutes. After reaction completion, the crude peptide was purified using a C4 semi-preparative column with a gradient of 0-60% B over 60 minutes to obtain (6.0 mg, 60 % yield).

Acm deprotection: 6 mg of desulfurized purified peptide was dissolved in 6M Gn·HCl, 200 mM phosphate buffer (pH 6.8) (final concentration 2 mM), was added 1.4 mg (15 eq.) of $PdCl_2$ and incubated at 37 °C for 30 minutes. Progress of the reaction was monitored by analytical HPLC using C4 analytical column and a gradient of 0–60% B over 30 minutes. After completion of the reaction, 60 eq. of DTT was added to quench and precipitate the palladium from the reaction mixture. After centrifugation, the supernatant solution was collected and purified using a C4 semi-preparative column with the gradient of 0-60% B over 60 minutes to obtain pure **7** (3.2 mg, 53% isolated yield).

Synthesis of dehydroalanine based probe SUMO2-V94DhaY (10) — Using 0.1 mmol from the previously split resin was used to complete the synthesis of the remaining residues (**Figure S7b**) following the general Fmoc-SPPS procedure. The crude peptide was then purified using preparative HPLC with a C4 column and a gradient of 15-50% B over 50 minutes, yielding peptide **10a** with an isolated yield of ~7%. 10 mg (8.70×10^{-4} mmol) of purified peptide **10a** was dissolved in 6 M Gn·HCl, 200 mM phosphate buffer (pH 8.30) (final concentration 2 mM). A solution of α, α''-di-bromo adipyl(bis)amide (26 mg, 100 eq) in 110 µl DMF was added to the peptide solution, and the reaction proceeded for 30 minutes at room temperature, followed by 2.5 hours at 37 °C.[5] The progress of the reaction was monitored by analytical HPLC using a C4 column and a gradient of 0-60% B over 30 minutes. After completion, the crude product was further purified

by semi-preparative HPLC using a C4 column with a gradient of 0-60% B over 60 minutes, yielding purified **10** (3.2 mg, ~19% yield).

20. Synthesis of SUMO2-EA (8)

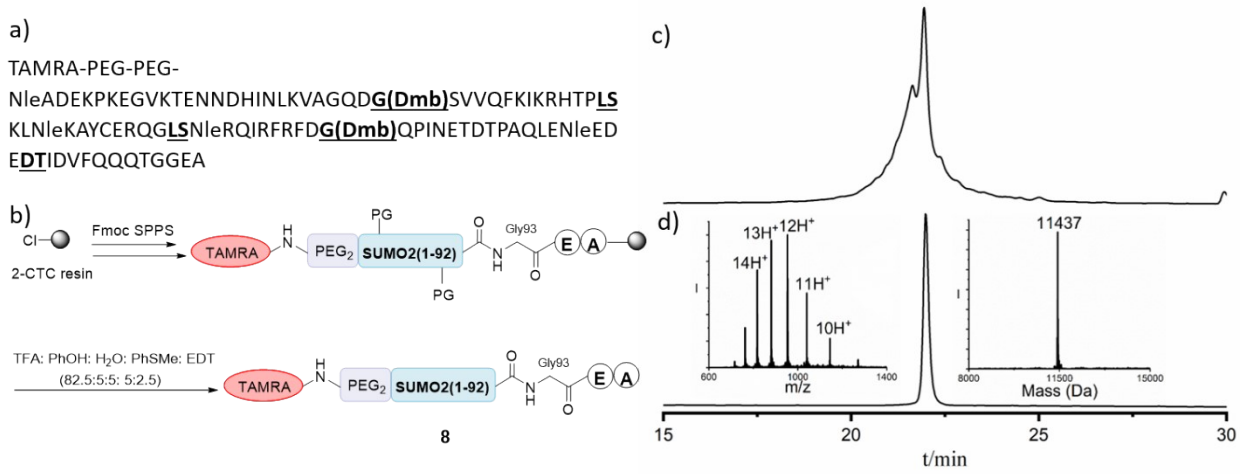


Figure S8. Synthesis of SUMO2-EA (**8**): a) Sequence of **8**. b) Schematic representation of synthetic route for **8**. c) Analytical HPLC chromatogram of crude **8** (detection at 214 nm). d) Analytical HPLC and mass analysis of purified **8** with the observed mass 11436.6 ± 0.6 Da, calcd. 11437.7 Da (average isotopes).

SUMO2-EA (**8**) was synthesized on 2-Chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Ala, as described in the general procedure of Fmoc-SPPS. The crude peptide **8** was purified by preparative HPLC using a C4 column with a gradient of 15-50% B over 50 minutes, yielding the corresponding peptide **8** with an isolated yield of ~8%.

21. Synthesis of dehydroalanine based probe SUMO2G94Dha-VY (9)

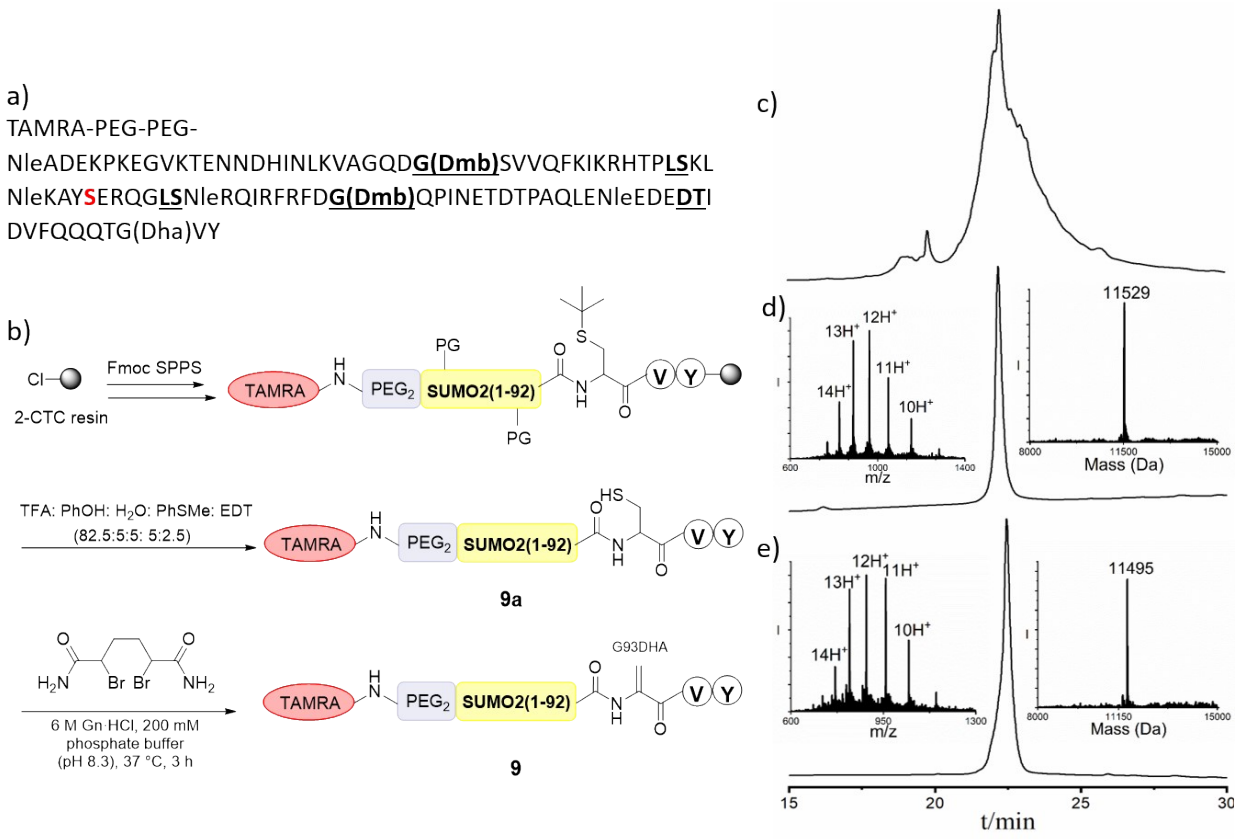


Figure S9. Synthesis of SUMO2G94Dha-VY (**9**): a) Sequence of **9**. (b) Schematic representation of synthetic route for **9**. (c) Analytical HPLC chromatogram of crude **9a** (detection at 214 nm). d) Analytical HPLC and mass analysis of purified **9a** with the observed mass 11529.4 ± 0.3 Da, calcd. 11530.9 Da (average isotopes). e) Analytical HPLC and mass analysis of purified **9** with the observed mass 11494.5 ± 0.5 Da, calcd. 11496.8 Da (average isotopes).

Peptide **9a** was synthesized on 2-Chlorotrityl chloride resin (0.2 mmol) pre-loaded with Fmoc-Tyr, as described in the general procedure of Fmoc-SPPS. The crude peptide **9a** was purified by preparative HPLC using a C4 column with a gradient of 15-50% B over 50 minutes, yielding the corresponding peptide **9a** with an isolated yield of ~6%. For subsequent reaction 10 mg (8.67×10^{-4} mmol) of purified **9a** was dissolved in 6M Gn·HCl, 200 mM phosphate buffer (pH 8.30) (final concentration 2 mM). A solution of α, α"-di-bromo adipyl(bis)amide reagent was prepared by dissolving 26 mg (100 eq) in 110 µl DMF.[5] This was added to peptide solution and the reaction was continued for 30 minutes at room temperature, followed by 2.5 h at 37 °C. The reaction progress was monitored by analytical HPLC using C4 analytical column and gradient of 0-60% B

over 30 minutes. After completion the crude was purified by semi-preparative HPLC using a C4 column with a gradient of 0-60% B over 60 minutes to obtain pure **9** (3.2 mg, ~22% isolated yield).

22. HPLC and ESI-MS analyses of SENP2 active domain and SUMO2 analogues

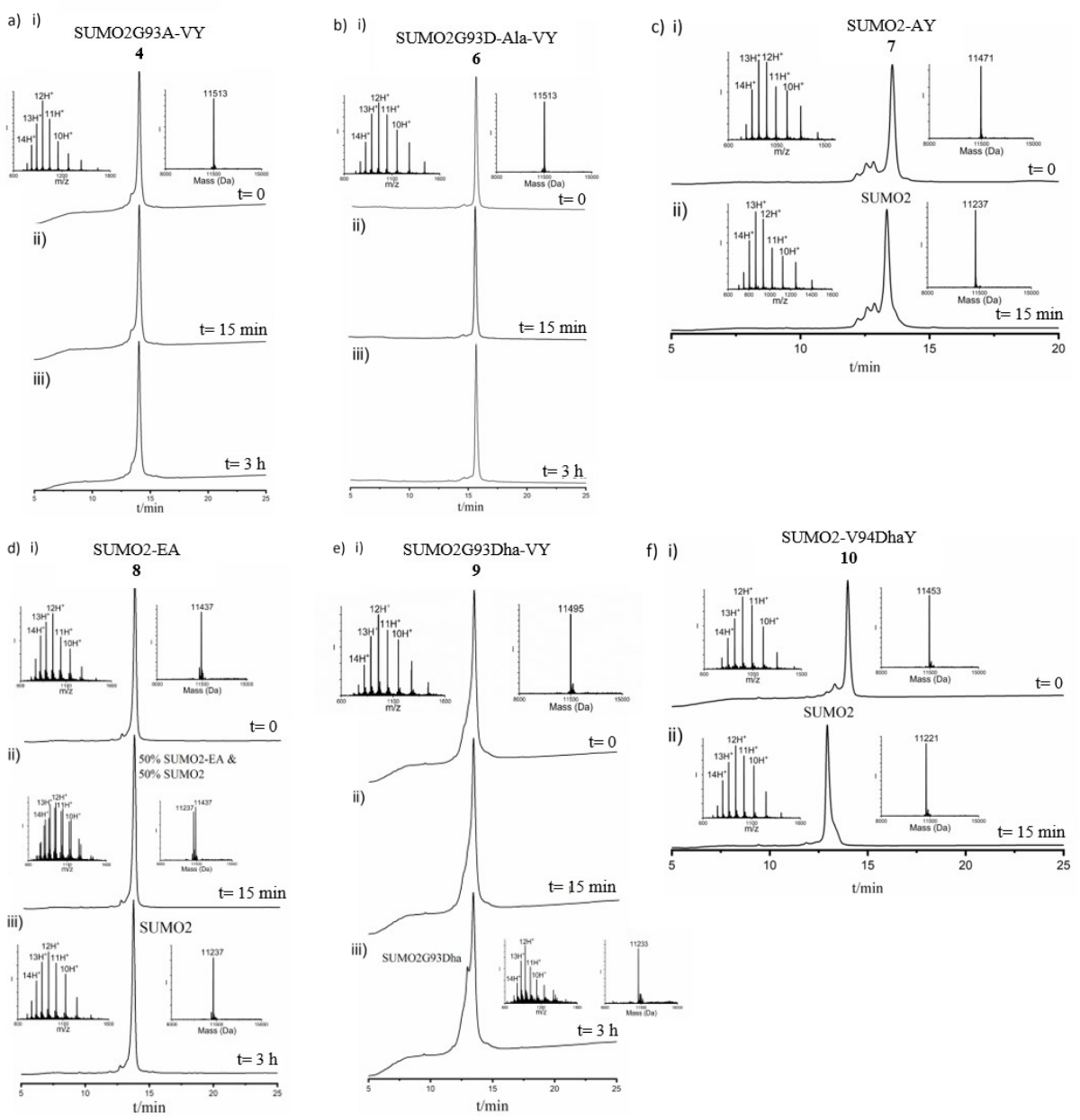


Figure S10. The *in vitro* reaction between SENP2 active domain and SUMO2 analogues **4, 6, 7-10**. i) t=0 represents SUMO2 analogue alone. (ii) t=15 min represents the reaction between the

analogue and SENP2 after 15 minutes of incubation. (iii) t=3 h represents the reaction between the analogue and SENP2 after 3 hours of incubation. a) The *in vitro* reaction with SUMO2G93A-VY (**4**). (i)-(iii) Observed mass 11512.8 ± 0.5 Da, calcd. 11514.9 Da. No change in mass was observed over time. b) The *in vitro* reaction with SUMO2G93D-Ala-VY (**6**). (i)-(iii) Observed mass 11512.8 ± 0.5 Da, calcd. 11514.9 Da. No change in mass was observed over time c) The *in vitro* reaction with SUMO2-AY (**7**). (i) Observed mass 11470.9 ± 0.5 Da, calcd. 11472.8 Da. (ii) Observed mass 11236.9 ± 0.4 Da, calcd. 11238.6 Da. d) The *in vitro* reaction with SUMO2-EA (**8**). (i) Observed mass 11436.5 ± 0.5 Da, calcd. 11437.7 Da. (ii) ~50% of the proteins with observed mass 11236.9 ± 0.4 Da, calcd. 11238.6 Da, and the other ~50% of the proteins with observed mass 11436.5 ± 0.5 Da, calcd. 11437.7 Da. (iii) Observed mass 11236.9 ± 0.4 Da, calcd. 11238.6 Da. e) The *in vitro* reaction with SUMO2G93Dha-VY (**9**). (i)-(ii) Observed mass 11494.9 ± 0.4 Da, calcd. 11496.8 Da. No change in mass was observed over time. (iii) Observed mass of the main peak 11494.9 ± 0.4 Da, calcd. 11496.8 Da, Observed mass of the minor peak 11232.9 ± 0.6 Da, calcd. 11234.5 Da. f) The *in vitro* reaction with SUMO2-V94DhaY (**10**). (i) Observed mass 11452.9 ± 0.6 Da, calcd. 11454.7 Da. (ii) Observed mass 11220.9 ±0 .4 Da, calcd. 11222.5 Da.

23. Live cell delivery of analogues 4-8, and PML-NBs studies

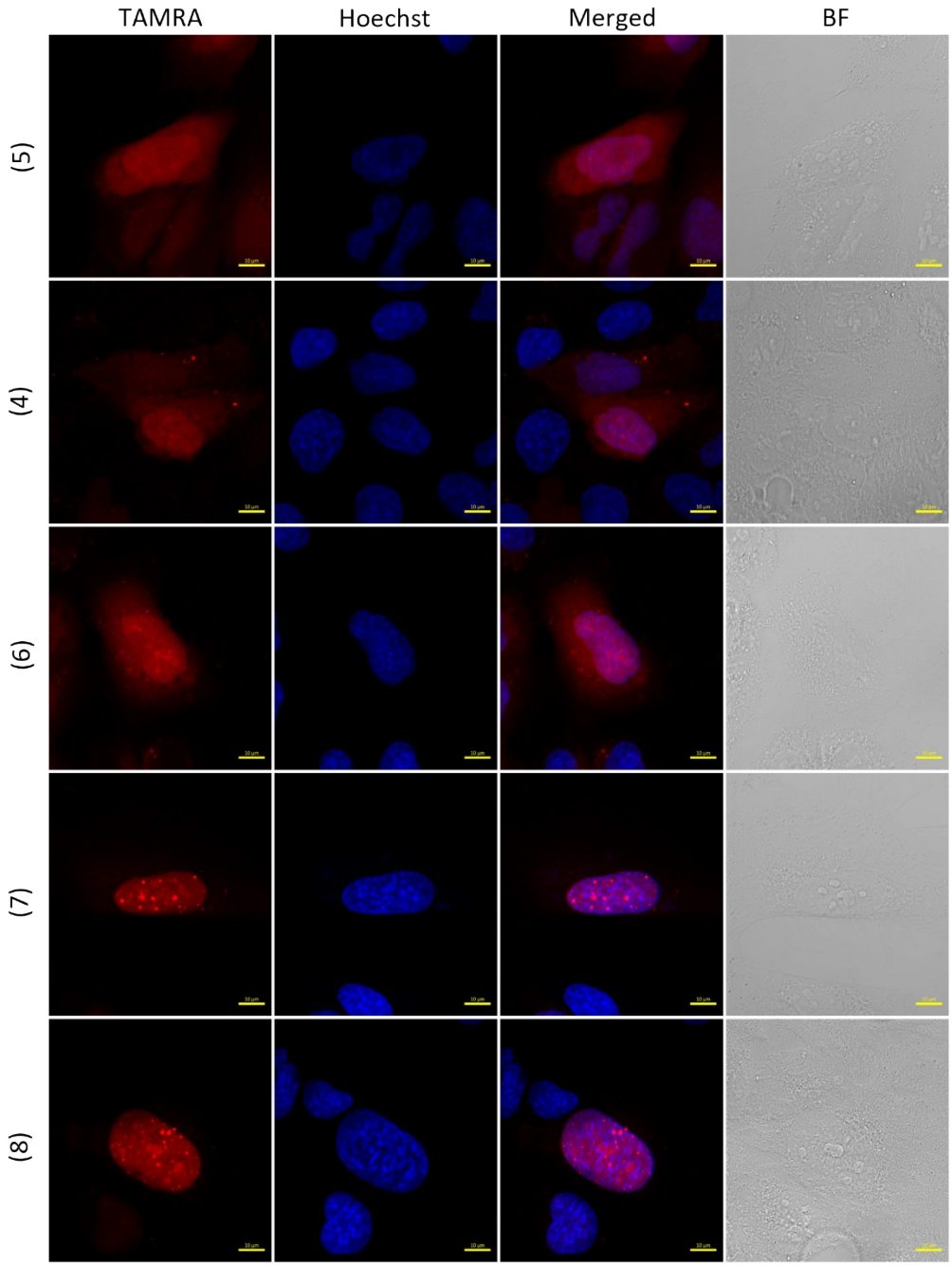


Figure S11. LSCM images of fixed U2OS cells loaded with 2 µM of **4**-**8** (TAMRA, red), and Hoechst nuclear stain (blue). Fixation was done 1 h after protein delivery. Scale bars are 10 µm. BF stands for Brightfield. Analogues **7** and **8** were observed in PML-NBs and analogues **4**-**6** were not observed in those compartments.

24. SDS-PAGE gels and western-blotting raw data

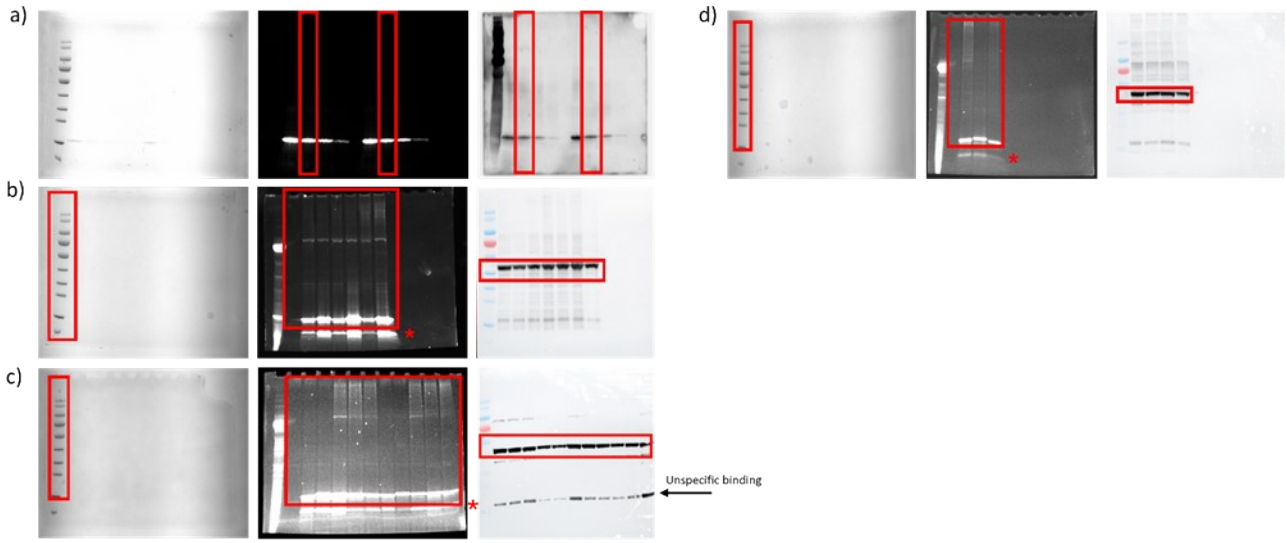


Figure S12. Full SDS-PAGE gels and Western blots. Cropped sections shown in the figures are marked. a) Full gel images of Figure 2c,d,g,h. b) Full gel images of Figure 3b. c) Full gel images of Figure 4b. d) Full gel images of Figure 5a.

*See Section 25.

25. Gel analyses for the synthetic and endogenous SUMO2

During gel analyses of cell lysates, two bands of our synthetic SUMO2 were observed. Because the synthetic protein alone runs as a single band in gel (Figure 2c-d, g-h, Figure S14 E-G), we hypothesized that degradation of the full-length protein (running at ~15 kDa) occurs during the cell lysis procedure leading to a truncated form of SUMO2 (running at ~10 kDa). To test this, we performed two different lysis protocols using two different lysis buffers, one was 2x Laemmli sample buffer and the other was RIPA buffer. 2X Laemmli sample buffer, the one we used in our experiments (mentioned in section 9), showed two bands of the endogenous SUMO2 protein (lane A). This indicates that the additional band observed is a result of the experimental procedure and not an impurity from the synthetic protein. When using RIPA buffer this extra band was not detected (lane B). It is worth noting that RIPA buffer yields faint bands of free SUMO2 and SUMO2 conjugates, meaning that this buffer is not compatible with our purposes to examine SUMO conjugates.

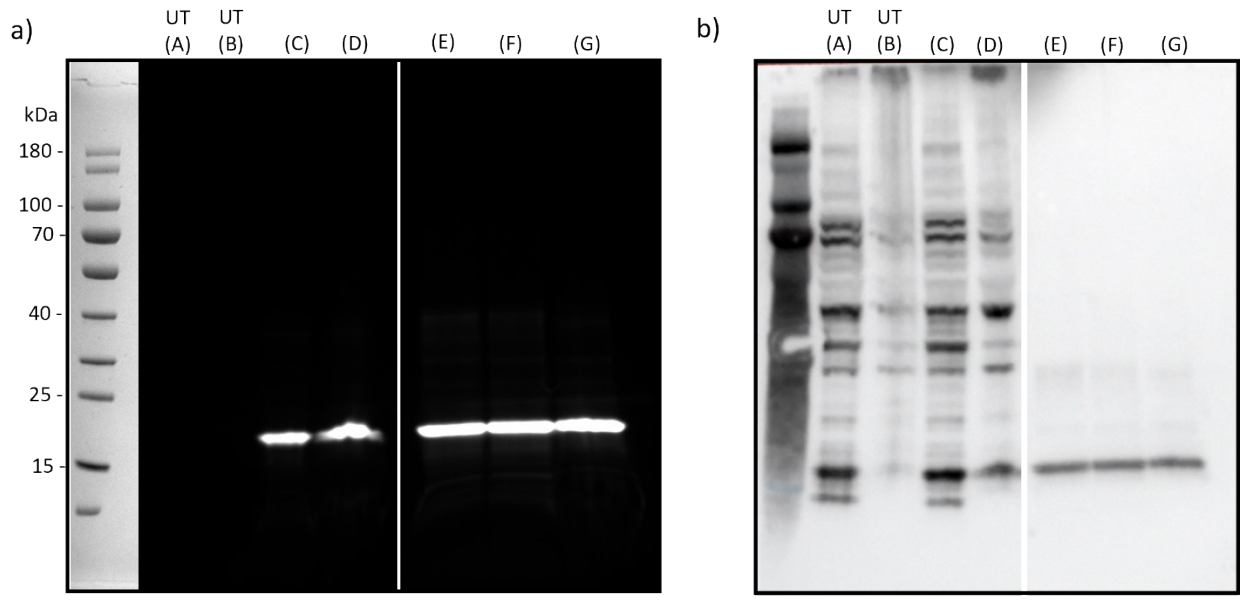


Figure S13. a) Fluorescence gel analyses of U2OS cell lysate (lanes A-D), and analogue **3** in buffers (lanes E-G) which was blotted in b) using SUMO2 antibody. Lane (A) represents untreated U2OS cells lysed with 2X Laemmli sample buffer. Lane (B) represents untreated U2OS cells lysed with RIPA buffer. Lane (C) represents untreated cells which were lysed in the addition of 1 µg of analogue **3** (using 2X Laemmli sample buffer). Lane (D) represents untreated cells which were lysed in the addition of 1 µg of analogue **3** (using RIPA buffer). Lane (E) represents only analogue **3** diluted in 2X Laemmli sample buffer. Lane (F) represents only analogue **3** diluted in 2X Laemmli sample buffer and proceeded with our lysis protocol. Lane (G) represents only analogue **3** diluted in RIPA buffer.

26. Lysis protocol using RIPA buffer

Cells were detached and pelleted as described in section 7. Then, the cell pellet was washed twice with cold PBS W/O Ca and Mg, and 100 µL of RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Deoxycholate, 0.1% SDS, 1% NP40, 1 µL of protease inhibitor cocktail) was added, followed by incubation on ice for 30 minutes, and centrifuge at 13,000 x g for 10 minutes at 4 °C. Final, the supernatant was collected and the lysis concentration was determined by Bradford assay against a BSA calibration curve.

27. References

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