Supporting Information

Total Chemical Synthesis of SUMO and SUMO-Based Probes for Profiling the Activity of SUMO-Specific Proteases

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Scheme S1. Rho-SUMOΔG-PA synthesis (PG = protecting group).

Figure S1. SDS-PAGE analysis of Rho-SUMOΔG-PA probes. M = marker, S1 = Rho-SUMO-1-PA, S2 = Rho-SUMO-2-PA, S3 = Rho-SUMO-3-PA, Ub = Rho-Ub-PA (control).
**Figure S2.** Labeling of the catalytic domains of SENP1 and SENP6 enzymes with Rho-SUMOΔG-PA probes. *M* = marker, *S1* = Rho-SUMO-1-PA, *S2* = Rho-SUMO-2-PA, *S3* = Rho-SUMO-3-PA, *Ub* = Rho-Ub-PA (control).
Figure S3. Activity-based profiling with SUMO ABPs. Labeling of ectopically expressed FLAG-SENP1-7 and FLAG-USP15 (negative control) in HeLa cell lysates A) visualized by fluorescence scan or B) immunoblot. S1 = Rho-SUMO-1-PA, S2 = Rho-SUMO-2-PA, S3 = Rho-SUMO-3-PA, Ub = Rho-Ub-PA (control), diS2 = K11 diSUMO-2-VA. Asterisks (*) indicates probe-labeled enzyme. Note: Additional bands with Rho-Ub-PA in the fluorescence scan are due to endogenous enzymes present in the lysate.
**Figure S4.** Labeling of overexpressed Flag-tagged SENP6 with Rho-SUMO-PA and K11 diSumo-2 probe. Dark exposure shows a preference of SENP6 for the SUMO -2 and -3 (S2/ S3) probes.

**Figure S5.** Analysis of K11 diSUMO-2-VA A) by SDS-PAGE and B) CD Spectrum.
Figure S6. Labeling of the catalytic domains of SENP1, 2, 5, 6, 7 and 8 with K11 diSUMO-2-VA. Asterisks (*) indicate probe-labeled SENP.

Figure S7. Time-dependent labeling of endogenous SENP1, SENP3 and SENP6 with A) SUMO-1-PA; B) SUMO-2-PA and C) diSUMO-2-VA. In contrast to the SUMO-1-PA and SUMO-2-PA probes, all of the tested SENPs readily react with the diSUMO-2-VA as depicted in C). Unlike SENP1, SENP3 appears to prefer diSUMO2-VA to SUMO-1-PA and SUMO-2-PA
**Figure S8.** Labeling of Flag-SENP1s with either Rho-SUMO2-PA, K11 diSUMO-2-VA, or the mono K11 α-amino-VA-SUMO-2 control probe. Transiently overexpressed Flag-SENP1, and Flag-SENP6 react with Rho-SUMO2-PA and K11-diSUMO-2-VA, but do not recognize the mono SUMO-2 K11 control probe bearing only the vinylamide electrophile. A) Schematic overview of the different types of ABPs used for the experiments. B) Labeling of ectopically expressed FLAG-SENP-1 and -6 visualized by immunoblot. An asterisks (*) indicates the probe-labeled enzyme. S2 = Rho-SUMO-2-PA, diS2 = K11 diSUMO-2-VA, Ctrl = K11 α-amino-VA-SUMO-2.
Figure S9. Quantification of the concentration of Rho-SUMO probes in cells after electroporation. A) Standard curve of 0.25-0.4 mg/mL Rho-SUMO-1-PA and Rho-SUMO-2-PA and the respective electroporation samples. B) Corresponding calibration curves of the Rho-SUMO probes. Corresponding band areas quantified from the in-gel fluorescence scans (A), listed in tables C) and D) indicate that approximately 82% of Rho-SUMO-1-PA and 93% of Rho-SUMO-2-PA are incorporated into the cells.
**Figure S10.** Differential regulation of endogenous SENPs. SENP1 and SENP3 versus SENP6 are inactivated in response to heat-shock.\(^1\) Cells from control (no HS) or heat-shocked (1h HS) were treated with either Rho-SUMO-2-PA (for SENP1 and SENP3) or K11 diSUMO-2-VA (for SENP6). SENP reactivity was assessed in the control (no HS), after heat-shock (1h HS) and recovery after heat-shock (2h after HS) samples. Unmodified SENPs as well as their ABP adducts are indicated and quantification of the SDS-PAGE bands of ABP adducts are shown in B).
General procedures

Fmoc SPPS strategy
SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 40 μmol scale using fourfold excess of amino acids relative to pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g, Applied Biosystems®). All amino acids and dipeptide building blocks were double coupled in NMP for 45 min using PyBOP (4 equiv) and DiPEA (8 equiv) as coupling regents. The following protected pseudoproline and DMB dipeptide building blocks were used during the SUMO synthesis as indicated in Figure 1: Fmoc-L-Ser(Bu)-L-Thr(ψMe,Me pro)-OH, Fmoc-L-Ser-L-Ser(ψMe,Me pro)-OH, Fmoc-L-Leu-L-Ser(ψMe,Me pro)-OH, Fmoc-L-Asp-L-Thr(ψMe,Me pro)-OH, Fmoc-L-Asp(OfBu)-(Dmb)Gly-OH. All amino acid and dipeptide building blocks were dried overnight under high vacuum prior to use. Fmoc removal was achieved with 20% piperidine in NMP (3×1.2 mL, 2×2 and 1×5 min). After completion of all coupling cycles the resin was washed with Et2O, dried under high vacuum and stored for further use. An initial trial cleavage using a small amount of the resin was performed to access the quality of the crude proteins (Analysis can be found in Figure S11).

Cleavage from the resin and deprotection
The polypeptide sequence was detached from the resin and deprotected by treatment with TFA/H2O/TiS/Phenol 90:5:2.5:2.5 v/v/v/v for 3 h followed by precipitation with cold Et2O/n-pentane 3:1 v/v. The precipitated protein was washed twice with Et2O and was finally lyophilized from H2O/CH3CN/CH3COOH 65:25:10 v/v/v.

LC-MS analysis
LC-MS measurements were performed on a system equipped with a Waters 2795 Seperation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750nm), Phenomenex Kinetex C18 (2.1x50, 2.6 μm) column and LCT™ ESI Mass Spectrometer. Samples were run using 2 mobile phases: A = 1%, CH3CN 0.1% formic acid in H2O and B = 1% H2O, 0.1% formic acid in CH3CN, at a flow rate of 0.8 mL/min. Gradient: 0–0.5 min, 5 % B; 0.5–8 min, →95 % B; 8–10 min 95% B, 10–12
min, → 5% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function).

**Preparative HPLC purification**

Preparative HPLC was performed on a Waters Auto Purification preparative HPLC-MS system, equipped with a Waters 2489 UV/Vis detector, Waters 2535 Quaternary Gradient Module, Waters Fraction collector III and a Xbridge C18 (10 mm x 150 mm, 5 μm) column. Samples (up to 20 μmol peptide per run) were run using 3 mobile phases: Mobile phases: A = H₂O, B = CH₃CN and C = 1% TFA in H₂O, at a flow rate of 37.5 ml/min. Gradient: 0–5 min, 5% B; 5-7 min, → 20% B; 7–25 min, → 60% B; 25–27 min, → 95% B; 27-32 min, 95% B; 32-33 min, 5% B. 0-33 min: D = 5%.

**Dissolving and folding of synthetic SUMO conjugates.**

The SUMO protein is dissolved in a minimal amount of warm DMSO at a concentration of 40-60 mg/ml. Then refolding was done by dropwise addition of the DMSO stock to water or buffer of choice. The final DMSO concentration is kept as low as possible (2 – 5%).

**CD measurements**

The structural conformation of our synthetic SUMO constructs were validated using circular dichroism (CD). The samples were dissolved in DMSO at a concentration of 50 mg/ml. Refolding was done by dissolving 10 μl of this DMSO stock in 2.5 ml MQ water. After a brief vortex, 2.5 ml of 20 mM sodium phosphate buffer (pH 7.0) was added to this solution. To remove DMSO from the sample, we used 3 kDa cut-off Amicon Ultra-15 Centrifugal Filter Units and exchanged the solvent for 20 mM sodium phosphate buffer (pH 7.0). Complete removal of DMSO was followed by the disappearance of DMSO peak in LC/MS. Final concentrations of the SUMO conjugates were measured using standard BCA assay. The CD measurements were made using JASCO CD J1000 series instrument at 25 °C. Samples were diluted in 20 mM sodium phosphate buffer (pH 7.0) so that the final concentration was approximately 10 μM. The CD spectra was recorded from 250 nm to 190 nm at a scan rate of 20 nm per minute and a scan width of 1 nm. A quartz cuvette with a path length of 1 mm was used in all measurements. In total, 8 cumulative measurements were made and the average was calculated and plotted using Graphpad PRISM.
Synthesis of SUMO

SUMO-1
The synthesis was performed following the general procedure. The crude product was purified by preparative HPLC as described in the general procedure. LC-MS: Rt 4.30 min; ESI MS+ (amu) calcd: 11130, found 11130 (deconv.).

SUMO-2
The synthesis was performed following the general procedure. The crude product was purified by preparative HPLC as described in the general procedure. LC-MS: Rt 4.23 min; ESI MS+ (amu) calcd: 10607, found 10607 (deconv.).

SUMO-3
The synthesis was performed following the general procedure. The crude product was purified by preparative HPLC as described in the general procedure. LC-MS: Rt 4.20 min; ESI MS+ (amu) calcd: 10523, found 10523 (deconv.).

SUMO-1\(\Delta\)G
The synthesis was performed following the general procedure. The methionine and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. After completing all coupling cycles, 687 mg resin was obtained. For analysis, 154 mg of this resin was weighed and used for detachment and deprotection of the polypeptide product. The crude product was purified by preparative HPLC as described in the general procedure. The final product was lyophilized and obtained as a white solid (6 mg, 10%); LC-MS: R, 6.34 min; ESI MS+ (amu) calcd: 10986, found 10988 (deconv.).

SUMO-2\(\Delta\)G
The synthesis was performed following the general procedure. The methionine and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. After completing all coupling cycles, 792 mg resin was obtained. For analysis, 154 mg of this resin was weighed and used for detachment and deprotection of the polypeptide product. The crude product was purified by preparative HPLC as described in the
general procedure. The final product was lyophilized and obtained as a white solid (17 mg, 21%); LC-MS: $R_t$ 4.38 min; ESI MS+ (amu) calcd: 10462, found 10462 (deconv.).

**SUMO-3ΔG**

The synthesis was performed following the general procedure. The methionine and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. After completing all coupling cycles, 704 mg resin was obtained. For analysis, 153 mg of this resin was weighed and used for detachment and deprotection of the polypeptide product. The crude product was purified by preparative HPLC as described in the general procedure. The final product was lyophilized and obtained as a white solid (27 mg, 30%); LC-MS: $R_t$ 4.32 min; ESI MS+ (amu) calcd: 10379, found 10378 (deconv.).
Preparation of SUMO probes

**Rho-SUMOΔG-PA probes**
The SUMOΔG peptide sequence was synthesized on a trityl resin following the general procedure. The methionine and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. After removal of the N-terminal Fmoc group, the resin was treated with a solution of Boc₂Rho (4 equiv, 160 µmol, 92 mg), PyBOB (4 equiv, 160 µmol, 83 mg) and DIPEA (8 equiv, 320 µmol, 56 µL) in NMP (5 mL). The mixture was gently shaken for 3h at room temperature before the resin was filtered and washed with NMP, DCM and Et₂O. Then, the resin bound polypeptide was treated with 5 mL of DCM/HFIP 7:3 v/v for 15 min and filtered. This DCM/HFIP treatment was repeated once before the resin was rinsed with DCM (3 × 5 mL). The combined filtrates were concentrated, co-evaporated with DCM and dried under high vacuum. The partially protected peptide residue (1 equiv) was redissolved in DCM and reacted with PyBOP (5 equiv, 200 µmol, 102 mg), propargyl amine (10 equiv, 400 µmol, 26 µL) and TEA (20 equiv, 800 µmol, 111 µL). The reaction mixture was stirred over night at room temperature. After removal of the solvent *in vacuo*, the residue was treated with 5 mL of TFA/H₂O/TlS/Phenol 90.5:5:2.5:2.5 v/v/v/v for 3 h followed by precipitation with cold MTBE/hexane 3:1 v/v. Further workup including lyophilization and purification by preparative HPLC were performed according to the general procedures.

**Rho-SUMO-1ΔG-PA**
The product (24 mg, 5%) was obtained as an orange solid. LC-MS: Rₜ 4.33 min; MS ESI MS⁺ (amu) calcd: 11380; found 11381 (deconv.). [M+H]⁺.

**Rho-SUMO-2ΔG-PA**
The product (45 mg, 10%) was obtained as an orange solid. LC-MS: Rₜ 4.28 min; MS ESI MS⁺ (amu) calcd: 10857; found 10857 (deconv.).

**Rho-SUMO-3ΔG-PA**
The product (44 mg, 10%) was obtained as an orange solid. LC-MS: Rₜ 4.28 min; MS ESI MS⁺ (amu) calcd: 10773; found 10773 (deconv.).
Preparation K11 diSUMO-2-VA probe

SUMO-2 K11Dab mutant
The SUMO-2 peptide sequence with K11 to Dab(Alloc) mutation was synthesized on Fmoc-Gly pre-loaded Wang resin following the general procedure. The methionine (Except for the final amino acid, here Boc protected methionine was used) and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. The resin was treated with Pd(PPh$_3$)$_4$ (0.35 eq) and Ph$_3$SiH (20 eq) in DCM (2x 20 min) and shaken overnight at room temperature with 4-((tert-butoxycarbonyl)amino)-3-(tert-butyldisulfaneyl)butanoic acid (3 eq), PyBOP (3 eq) and DiPEA (6 eq). After extensive washings (3x NMP & DCM & Et$_2$O) the resin was treated with TFA/H$_2$O/phenol/iPr$_3$SiH (90/5/2.5/2.5 v/v/v/v) for 3 h followed by precipitation in cold Et$_2$O/pentane 3:1 v/v. The crude peptide was lyophilized and purified by preparative HPLC as described in the general procedure. SUMO-2 K11Dab(1): LC-MS: R$_t$ 6.34 min; ESI MS+ (amu) calcd: 10715, found 10717 (deconv.).

SUMO-2ΔG-SEt
The SUMO-2ΔG peptide sequence was synthesized on a trityl resin following the general procedure and using Boc protected norleucine as final amino acid (40 μmol). The methionine and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. The resin was treated with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filtered. The resin was rinsed with DCM (3x 5 mL) and the combined filtrates were concentrated. The partially protected peptide residue (1 equiv) was redissolved in DCM and reacted with pyBOP (5 equiv), EtSH (10 equiv) and DiPEA (10 equiv). The reaction mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue treated with TFA/H$_2$O/phenol/iPr$_3$SiH (90/5/2.5/2.5 v/v/v/v) for 3 h followed by precipitation with cold Et$_2$O/pentane 3:1 v/v. Purification by preparative HPLC as described in the general procedure gave SUMO-2ΔG-SEt as a white powder. LC-MS: R$_t$ 6.40 min; ESI MS+ (amu) calcd: 10506; found 10508 (deconv.).
Chemical ligation under denaturing conditions.
To a solution of the SUMO-2 K11Dab mutant in 0.2 M sodium phosphate buffer (pH 7) containing 6 M Gdn-HCl and MPAA (250 mM), a solution of SUMO-2ΔG-SEt (1.2 equiv) in 0.2 M sodium phosphate buffer (pH 7) containing 6 M Gdn-HCl and MPAA (250 mM) was added and the mixture (conc: 50 mg/mL) was incubated at 37°C. After incubation at 37°C overnight, TCEP was added to reduce the MPAA disulfide. The obtained mixture was purified by preparative HPLC as follows: Mobile phases: A = H2O, B = CH3CN and C = 1% TFA in H2O. Xbridge C18 (10x150mM, 5 μm); flow rate = 37.5 ml/min. Gradient: 0–5 min, 5% B; 5-7 min, →20% B; 7–25 min, →95% B; 25-30 min, 95% B; 30-31 min, 5% B. 0-31 min: D = 5%. Pure fractions were pooled and lyophilized. ESI MS+ (amu) calcd: 21071; found 21083 (deconv., oxidized methionine).

Thiol elimination of the warhead - K11 diSUMO-2-VA
To a solution of the K11-linked precursor in 50 mM sodium phosphate buffer (pH 8, 0.5 mg/mL) 2,5-dibromohexandiamide (100 eq.) was added. The reaction mixture was incubated at 37°C overnight and spun down to remove the insoluble dibromide. The obtained mixture was purified by preparative HPLC as follows: Mobile phases: A = H2O, B = CH3CN and C = 1% TFA in H2O. Xbridge C18 (10x150mM, 5 μm); flow rate = 37.5 ml/min. Gradient: 0–5 min, 5% B; 5-7 min, →20% B; 7–25 min, →95% B; 25-30 min, 95% B; 30-31 min, 5% B. 0-31 min: D = 5%. Pure fractions were pooled, lyophilized and run over size exclusion S75 column. LC-MS: Rf 4.5 min; ESI MS+ (amu) calcd: 21037; found 21035 (deconv.).
Correct folding of the purified synthetic diSUMO-2-VA was verified by CD spectroscopy (see general procedure page S10 and Figure S5).

Mono control probe: K11 SUMO-2 ABP
The SUMO-2 peptide sequence with K11 to Dab(Alloc) mutation was synthesized on Fmoc-Gly pre-loaded Wang resin following the general procedure. The methionine (except for the final amino acid, here Boc protected methionine was used) and cysteine residues in the sequence were replaced by the known isosters norleucine and serine, respectively that do not interfere with normal function of the protein. The resin was treated with Pd(PPh3)4 (0.35 eq) and Ph3SiH (20 eq) in DCM (2x 20 min) and shaken overnight at room temperature with 4-(((allyloxy)carbonyl)amino)but-2-
enoic acid (4 eq), PyBOP (4 eq) and DiPEA (8 eq). The resin was treated with Pd(PPh₃)₄ (0.35 eq) and Ph₃SiH (20 eq) in DCM (2x 20 min) to remove the Alloc protecting group. TFA/H₂O/phenol/iPr₃SiH (90/5/2.5/2.5 v/v/v/v) for 3 h followed by precipitation in cold Et₂O/pentane 3:1 v/v. After extensive washings (3x NMP & DCM & Et₂O) the resin was treated with TFA/H₂O/phenol/iPr₃SiH (90/5/2.5/2.5 v/v/v/v) for 3 h followed by precipitation in cold Et₂O/pentane 3:1 v/v. The crude peptide was lyophilized and purified by preparative HPLC as described in the general procedure. K11 SUMO-2 ABP: LC-MS: Rt 4.81 min; ESI MS+ (amu) calcd: 10592; found 10592 (deconv.).
Procedures for labeling experiments

Cell culture
HeLa and HEK293T cells obtained from the ATCC were cultured under standard conditions in DMEM (Life Technologies) supplemented with 10% FCS (Sigma Aldrich) at 37°C and 5% CO₂.

Buffers
Sucrose based TRIS lysis buffer: 50 mM TRIS pH 7.4, 250 mM sucrose, 5 mM MgCl₂, protease inhibitor, 1 mM DTT and 2 mM ATP. DTT and ATP were freshly added to the lysis buffer prior to use.
RIPA lysis buffer: 50 mM TRIS (pH 7.4), 1% Triton-X 100, 0.5% Sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 2 mM ATP. DTT and ATP were freshly added to the lysis buffer with each preparation.
Electroporation buffer: 2 mM HEPES, 15 mM K₂HPO₄/KH₂PO₄ pH 7.2, 1 mM MgCl₂, 250 mM mannitol.

Visualization of Rho-SUMO conjugates
Labeled enzymes were visualized by in-gel fluorescence using either the ProXPRESS 2D Proteomic imaging system (Perkin-Elmer) with a resolution of 100 μm and exposure time of 20 s, or using the Typhoon FLA imaging system (GE Healthcare Life Sciences) (λₑₓ/λᵦₘ 480/530 nm). Subsequently, gels were stained with Coomassie (Coomassie Brilliant Blue) and proteins were visualized using a Chemidoc XRS+System with Image Lab software (Bio-Rad).

SENP1 and SENP6 labeling
The activity of the synthesized Rho-SUMO-PA probes was verified using purified catalytic domains of the SENPs. To label the proteases, GST-SENP1 or GST-SENP6 (1.2 μg total protein) was incubated with Rho-SUMO-1, -2, -3-PA or Rho-Ub-PA (0.64 μg total probe each) in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4) for 30 minutes at RT. The reaction was terminated by the addition of 3x SDS-PAGE Loading Buffer (Invitrogen) containing beta-mercaptoethanol. Samples were resolved by SDS-PAGE (4-12% NuPage gel) and visualized by in-gel fluorescence scanning followed by staining with Coomassie Brilliant Blue.
SENP labeling with K11 diSUMO-2-VA

SENP labeling with K11 diSUMO-2-VA probe from a stock solution of 2 mg/ml. The reaction was incubated at 37°C for 1 hour with constant shaking. The reaction was terminated by the addition of 3x SDS-PAGE Loading Buffer (Invitrogen) containing beta-mercaptoethanol. Samples were resolved by SDS-PAGE (4-12% Invitrogen NuPage gel) and visualized by staining with Coomassie Brilliant Blue.

Labeling of endogenous enzymes in cell lysates

Cell lysates were prepared by resuspending cell pellets in 3 pellet volumes of HR buffer (50 mM TRIS, pH 7.4, 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT) and lysed by sonication. After clarification by centrifugation (20,000 rpm, 4°C, 20 min), total protein concentration was determined by Nanodrop.

For the labeling experiments, 600 μg of lysate was incubated with 4 μg Rho-SUMO-1, -2, -3-PA and Rho-Ub-PA in Labeling Buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) at RT for 15 min. The reaction was terminated by the addition of 3x SDS-PAGE Loading Buffer (Invitrogen) containing beta-mercaptoethanol. Samples were resolved by SDS-PAGE and visualized by fluorescence scanning (λex = 625 nm; λem = 680 nm). For visualizing the reactivity of endogenous SENP1, SENP3, and USP7 western blotting was performed as previously described and membranes were probed with rabbit SENP1 (1:1000 dilution; Epitomics (Abcam), 28871), rabbit SENP3 antibody (1:1000 dilution; Cell Signalling Technology, 5591), USP7 or mouse anti-β-actin (1:10000 dilution; Sigma A544), as indicated. Fluorescent secondary antibodies anti-mouse-800 (1: 10000 dilution; LiCOR, 926-3210) and anti-rabbit-800 (1: 10000 dilution; LiCOR, 926-3211) were used for visualization of labeled proteins on LICOR Odyssey system v3.0.
Labeling of overexpressed SENPs in cell lysates

For overexpression of epitope-tagged SENPs, HELA cells were transiently transfected with Flag-SENP1, Flag-SENP2, Flag-SENP3, Flag-SENP6 and Flag-SENP7. As an additional control, human Flag-USP15 was included. Human Flag-USP15 was subcloned from pDEST-cDNA constructs (Addgene) into 2xFLAG-C1 vector (Clontech) at HindIII/XbaI and XhoI/EcoRI respectively. DNA was delivered into HEK293T cells using polyethylenimine (PEI, Polysciences, Inc.) according to manufacturer’s instructions. 24h following transfection, cells were harvested by scraping in lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 0.5% Triton-X100, Protease Inhibitor tablet (Roche)) and lysate was clarified by centrifugation. Reactions (30 μl of lysate) were incubated in the presence of indicated components with Rho-SUMO-1, -2, -3-PA, Rho-Ub-PA, K11 diSUMO-2-VA or control probe K11 SUMO2 ABP (2.0 μg/reaction), stationary for 15 min at RT. Reactions were stopped by the addition of sample loading buffer containing beta-mercaptoethanol, followed by boiling for 10 min. Samples were resolved using standard SDS-PAGE, and probe reactivity was assessed by in-gel fluorescence scanning, followed by transfer onto nitrocellulose membranes and immunoblotting using mouse anti-Flag (1:1000 dilution; Sigma Aldrich, F3165), rabbit anti-SENP2 (1:1000 dilution; Bethyl, A303-016A) or mouse anti-β-actin (1:10000 dilution; Sigma Aldrich, A544), as indicated. Fluorescent secondary antibodies anti-mouse-800 (1: 10000 dilution; LiCOR, 926-3210) and anti-rabbit-800 (1: 10000 dilution; LiCOR, 926-3211) were used for visualization of labeled proteins on LICOR Odyssey system v3.0.

Time-dependent labeling of SUMO-specific proteases

To investigate the reactivity of SUMO-specific proteases in a time-dependent manner, 4 μg of Rho-SUMO-1-PA, Rho-SUMO-2-PA, or diSUMO-2-VA were added to 250 μg Hela cell lysate, prepared as previously described. After incubation for the indicated amount of time, 30 μL of sample was removed from the reaction and quenched with 3x SDS-PAGE loading buffer (NuPAGE, Invitrogen). After resolving by SDS-PAGE gel electrophoresis using 10% Bis-Tris gels (Invitrogen) and subsequent transfer to nitrocellulose membranes, reactivity of endogenous SENPs was visualized by probing the membranes with the following antibodies: anti-rabbit SENP1 (1:1000 dilution; Epitomics (Abcam) 28871), rabbit anti-SENP3 (1:1000 dilution; Cell
Signaling Technology, 5591), mouse anti-SENP6 (1:1000 dilution, SIGMA, WHP0026054M1) and anti-mouse beta-actin antibodies (1:10000 dilution; Sigma, A2228). Fluorescent secondary antibodies anti-mouse-800 (1: 10000 dilution; LiCOR, 926-3210) and anti-rabbit-800 (1: 10000 dilution; LiCOR, 926-3211) were used for visualization of labeled proteins on LICOR Odyssey system v3.0.

SENP reactivity after heat-shock.
HEK293 cells, stably expressing a tetracyclin-inducible constitutive dominant negative HSF1, were exposed to heat stress (HS) for 75min at 43°C and subsequently incubated for 2 hours at 37°C for recovery. Cell lysates were prepared by resuspending cell pellets in 2 pellet volumes of lysis buffer (containing 0.25 M sucrose, 20 mM MOPS-KOH, 1 mM EDTA-NaOH, 1 tablet/10 ml completeTM Mini protease inhibitor cocktail (Sigma), 1 mM DTT, pH 7.4). and lysed by sonication. After clarification by centrifugation (20,000 rpm, 4°C, 5 min), total protein concentration was determined by Nanodrop.

100 µl of supernatant (~200 µg total protein) was labelled with either 8 µg of Rho-SUMO-2-PA or K11 di-SUMO-2-VA for 30 min at 37°C. The reaction was terminated by the addition of NuPage LDS sample buffer (Invitrogen). Samples were resolved by SDS-PAGE on Novex Bolt 4-12% Bis-Tris Plus gels (for SENP1 and SENP3) or 3-8% Tris-Acetate gels (SENP6). For visualizing the reactivity of endogenous SENP1, SENP3, and SENP6 western blotting was performed as previously described and membranes were probed with rabbit SENP1 (1:1000 dilution; Epitomics (Abcam), 28871), rabbit SENP3 antibody (1:1000 dilution; Cell Signalling Technology, 5591), or mouse SENP6 antibody (1:1000 dilution, SIGMA, WHP0026054M1), as indicated. Fluorescent secondary antibodies anti-mouse-800 (1: 10000 dilution; LiCOR, 926-3210) and anti-rabbit-800 (1: 10000 dilution; LiCOR, 926-3211) were used for visualization of labeled proteins on LICOR Odyssey system v3.0.
Activity-based profiling in living cells

Electroporation of SUMO-1 and SUMO-2 into living cells
For confocal experiments, confluent HeLa cells (80,000 cells) were seeded in a 6-well plate and transfected with Flag-SENP1, Flag-SENP2 and Flag-SENP7 using Effectene (Qiagen), according to manufacturer’s instructions. To facilitate the incorporation of the probe, the growth medium was refreshed 4-6 hours following transfection and again 1-2 hours prior to electroporation. After removing the growth medium, cells were kept on ice throughout the entire electroporation procedure and washed twice with ice-cold electroporation buffer. For incorporating the probes, 2.0 mL of a solution of Rho-SUMO-1-PA or Rho-SUMO-2-PA (0.4 mg/mL, 0.5% DMSO) dissolved in electroporation buffer was added to the cells and electroporated using a Biorad GenePulser Xcell with CE and PE module Pulse Generator equipped with a Petri Pulser electroporation applicator (BTX) using the following settings: square wave, voltage=75 V, pulse length=3 ms, pulse interval=1.5 s, number of pulses=5, cuvette width=2 mm. The electroporation applicator was turned 90 degrees, and electroporation was repeated once. Following electroporation, the cells were allowed to recover on ice in cold electroporation buffer for 2 min before being washed twice with ice-cold PBS. Subsequently, treated cells were allowed to recover under standard growth conditions as indicated (120 min).

Quantification of probe electroporated into living cells
To quantify the amount of Rho-SUMO-PA actually entering the cells after electroporation, a standard curve with Rho-SUMO-1-PA or Rho-SUMO-2-PA ranging from 0.25-0.4 mg was generated. After in-gel fluorescence scanning, the fluorescence signal from the samples of cells electroporated with the SUMO-probes and the calibration curves was quantified using ImageQuant TL 8.1 Software (GE Healthcare Life Sciences). Calculation of the percentage of probe entering the cells indicates that about 82% of Rho-SUMO-1-PA and 93% of Rho-SUMO-2-PA are incorporated (Figure S8).
**Confocal microscopy**

For microscopy experiments, samples were fixed using 4% paraformaldehyde (Merck) in PBS and mounted on glass cover slips (Thermo Scientific) using Prolong Gold mounting medium containing DAPI (Invitrogen). Image acquisition was performed collecting z stacks of 5 images per cell on a Leica SP5 or SP8 confocal microscope equipped with HyD detectors, oil immersion lens, a digital zoom of 2.5-4x. Image processing was performed using Image J software and the resulting images are represented as maximum z projections.
LC-MS analysis of synthesized proteins

Figure S11. Analysis of crude SUMO proteins directly after SPPS. Diode array chromatogram (left) and MS spectrum (right). A) SUMO-1ΔG; B) SUMO-2ΔG; C) SUMO-3ΔG.
Figure S12. LC-MS analysis of SUMO-1 FL. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S13. LC-MS analysis of SUMO-2 FL. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S14. LC-MS analysis of SUMO-3 FL. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S15. LC-MS analysis of SUMO-1ΔG. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
**Figure S16.** LC-MS analysis of SUMO-2ΔG. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S17. LC-MS analysis of SUMO-3ΔG. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S18. LC-MS analysis of Rho-SUMO-1ΔG-PA. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S19. LC-MS analysis of Rho-SUMO-2ΔG-PA. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
**Figure S20.** LC-MS analysis of Rho-SUMO-3ΔG. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S21. LC-MS analysis of Rho-UbΔG-PA. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S22. LC-MS analysis of SUMO-2 K11 Dab mutant. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S23. LC-MS analysis of SUMO-2ΔG-SEt. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S24. LC-MS analysis of K11 diSUMO-2 precursor. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S25. LC-MS analysis of K11 diSUMO-2-VA. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
Figure S26. LC-MS analysis of mono control K11 SUMO2 ABP. Diode Array chromatogram (top). MS spectrum of peak (middle). Deconvulated mass of product peak (bottom).
References

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