



### **UbiQ protocol P004 \_SUMO protease activity assays**

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# Assays of SUMO protease/ isopeptidase activity and function in mammalian cells and tissues

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## Abstract

Covalent conjugation of the ubiquitin-related SUMO modifier to lysine residues of cellular proteins (SUMOylation) is a prevalent posttranslational modification. SUMOs are synthesized as precursor proteins that require carboxy-terminal processing prior to conjugation. Subsequently, a multistep enzymatic pathway is used for conjugation to target proteins. SUMOylation generally impacts protein–protein interactions and the assembly of multiprotein complexes. Cellular processes regulated by SUMOylation include DNA damage responses, cell cycle progression, or the control of gene expression. SUMOylation is reversible and commonly only a small fraction of a particular SUMO target is modified at a given time. Deconjugation of SUMO is catalyzed by a group of cysteine proteases termed SUMO proteases or SUMO isopeptidases. In human cells nine SUMO proteases, belonging to three separate families of cysteine proteases have been identified so far. The regulation and target specificity of individual SUMO proteases have not been dissected in detail, but the current view is that each protease

controls the modification of subsets of proteins that are functionally and/or physically linked. Importantly, some SUMO proteases/isopeptidases not only function in deconjugation of SUMO from proteins, but also act in C-terminal processing of the SUMO precursors. Here we describe general methods for monitoring SUMO protease/isopeptidase activities in cell or tissue extracts.



## 1. Introduction

Small ubiquitin-related modifier (SUMO) shares structural and sequence similarity with ubiquitin and belongs to the ubiquitin protein family (Flotho & Melchior, 2013; Gareau & Lima, 2010). Yeast and most non-vertebrates harbor a single SUMO (alias Smt3) gene, whereas vertebrates typically have genes for more than one SUMO isoform. In human tissues, three SUMO forms (SUMO1, SUMO2, and SUMO3) are ubiquitously expressed. Additionally, a tissue-specific SUMO4 form has been described, but its functional role has remained elusive (Bohren, Nadkarni, Song, Gabbay, & Owerbach, 2004). At the amino acid level, SUMO1 is about 50% identical with the nearly identical SUMO2 and SUMO3 isoforms, commonly denoted as SUMO2/3 (Muller, Hoege, Pyrowolakis, & Jentsch, 2001). SUMO1, -2, and -3 can be covalently attached to target proteins via an isopeptide linkage that connects the C-terminal glycine residue in SUMO with an  $\epsilon$ -amino group of a lysine residue in the target protein. Proteomic studies give an estimate of around 5000 SUMO sites in mammalian cells in culture (Hendriks et al., 2018). Under stress conditions this number increases to around 15,000 sites. All SUMO forms are synthesized as precursor proteins with extended C-termini that require proteolytic processing to expose the conjugation-proficient terminal glycine residue. Processing removes 4 (pre-SUMO1), 2 (pre-SUMO2), and 11 (pre-SUMO3) amino acids from the C-terminus of the respective pre-SUMOs. Pre-SUMO4 appears to be resistant to processing and therefore most likely cannot act as a modifier (Owerbach, McKay, Yeh, Gabbay, & Bohren, 2005).

Conjugation of processed SUMO proceeds by an enzymatic cascade involving the heterodimeric E1 enzyme (Aos1/Uba2), the E2 enzyme Ubc9, and a relatively small set of E3 SUMO ligases (Cappadocia & Lima, 2018). Attachment of SUMO to proteins typically fosters protein interactions by binding of the SUMO moiety to specific SUMO recognition motifs termed SUMO-interacting motifs (SIMs). SUMO can be conjugated

as a monomer on single or multiple sites on a target protein. Moreover, like ubiquitin (Ub), SUMO is able to form polymeric chains via internal lysine residues in SUMO. SUMO chains, in particular SUMO2/3 chains, are induced in response to cellular stress, such as redox, heat, or oncogenic stress, pointing to a critical role of SUMO chains in coping with these situations. The function of SUMO chains is best understood in the SUMO-targeted Ub ligase (STUbL) pathway where SUMO2/3 polymers are recognized by a distinct class of Ub E3 ligases that harbor tandem SIMs that bind preferentially to polySUMO chains (Ulrich, 2008; Vertegaal, 2010). The prototypical mammalian STUbL RNF4 is recruited to poly-SUMOylated proteins, triggering their proteolytic or nonproteolytic ubiquitylation.

All human SUMO proteases identified so far are cysteine proteases that can be subdivided into three classes: the SENP family with six members, the DESI (deSUMOylating isopeptidase) family with two members, and the unique USPL1. Compared to the Ub system, the SUMO deconjugation machinery is far less complex. Distinct SUMO proteases are thought to control the modification status of large sets of proteins. The best characterized class of SUMO isopeptidases/proteases is the Ulp/SENP family. They belong to the clan CE of cysteine proteases that is characterized by an adenain-like catalytic domain. Founding members of this family are the *Saccharomyces cerevisiae* Ulp1 and Ulp2 proteins (Li & Hochstrasser, 1999, 2000). The human SENP family is comprised of the six SUMO-specific SENPs (SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7) and the NEDD8-specific SENP8 (Hickey, Wilson, & Hochstrasser, 2012; Mukhopadhyay & Dasso, 2007). Phylogenetic analysis subdivides Ulp/SENP proteins into an Ulp1 and an Ulp2 branch, with human SENP1, SENP2, SENP3, and SENP5 belonging to the Ulp1 branch and SENP6 and SENP7 part of the Ulp2 branch. Sequence comparisons show higher similarity between certain pairs of human SENPs, in particular, SENP1-SENP2, SENP3-SENP5, and SENP6-SENP7 (Kunz, Piller, & Muller, 2018; Nayak & Muller, 2014).

SENPs catalyze two distinct proteolytic reactions. First, they act as processing enzymes for maturation of the pre-SUMO proteins by hydrolyzing a peptide bond near the carboxy terminus of the pre-SUMO; this leaves a SUMO C-terminus that ends with a pair of glycine residues. Second, they function as isopeptidases by cleaving the amide (isopeptide) bond that links SUMO moieties to the  $\epsilon$ -amino group of lysine residues. In vitro experiments indicate that distinct SENPs exhibit differential activities in processing pre-SUMO forms. SENP1 and SENP2 catalyze processing of all three precursors, but SENP2 is most active on SUMO2, whereas SENP1 prefers

SUMO1 over SUMO2 and SUMO3 (Reverter & Lima, 2004, 2006; Shen, Dong, Liu, Naismith, & Hay, 2006; Shen, Tatham, et al., 2006). SENP3 and SENP5 have a strong preference for pre-SUMO2 processing (Kolli et al., 2010; Mikolajczyk et al., 2007), while SENP6 and SENP7 are almost inactive in SUMO maturation (Kolli et al., 2010; Lima & Reverter, 2008). However, the *in vivo* contribution of a given SENP to the processing of a particular pre-SUMO is still not clear.

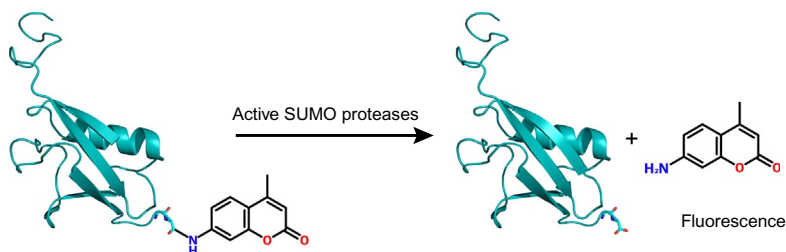
The isopeptidase activity of SENPs has been most thoroughly studied using SUMO-modified RanGAP1 as a model substrate (Kolli et al., 2010; Mikolajczyk et al., 2007; Reverter & Lima, 2006; Shen, Dong, et al., 2006; Shen, Tatham, et al., 2006). SENP1 and SENP2 deconjugate all SUMO isoforms from RanGAP1 with comparable efficiency, whereas SENP3 and SENP5 exhibit a strong preference for deconjugation of SUMO2/3 from RanGAP1 (Kolli et al., 2010; Mikolajczyk et al., 2007). SENP6 and SENP7 most efficiently cleave di- and polySUMO2/3 chains that are linked through internal lysine residues (Kolli et al., 2010; Lima & Reverter, 2008; Shen, Geoffroy, Jaffray, & Hay, 2009). The substrate specificity of distinct SENPs in a cellular context is not yet clear. Importantly, however, SENP family members exhibit a characteristic subcellular localization, indicating that their activities are constrained in a spatially regulated manner (Kunz et al., 2018; Nayak & Muller, 2014).



## **2. Activity measurements of deSUMOylating enzymes**

### **2.1 Monitoring SUMO protease activities by SUMO-AMC assays**

Substrates used in protease activity assays frequently rely on the covalent linkage of a fluorogenic dye to a peptide (Reverter & Lima, 2009). A common fluorophore is 7-amido-4-methylcoumarin (AMC). AMC is chemically ligated to the carboxy terminus of the protein of interest (in this case, SUMO) via a reactive amino group; this quenches the intrinsic fluorescence of AMC. When AMC is released by protease cleavage of the amide bond, the fluorescence intensity of the released dye is linearly proportional to the enzyme activity. To examine the activity of SUMO proteases, there are commercially available SUMO substrates in which the AMC moiety is coupled to the C-terminal diglycine motif of processed SUMO isoforms (SUMO1-AMC or SUMO2-AMC). When SUMO proteases cleave the linkage between AMC and SUMO, fluorescence of the released AMC molecule is measured using 380nm as excitation and 460nm as emission



**Fig. 1** Schematic view of SUMO protease assays using SUMO-AMC substrates. In the commercially available substrates, an AMC moiety is covalently linked to the C-terminal glycine of SUMO (SUMO1-AMC or SUMO2-AMC). SUMO protease cleavage of the bond between AMC and SUMO releases the fluorescent AMC molecule.

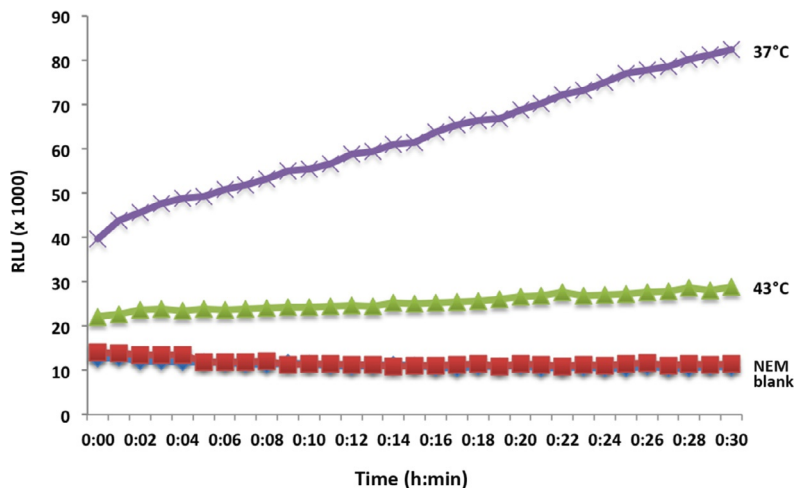
wavelengths (see Fig. 1 for reaction mechanism). This assay is sensitive and generates quantitative data that can be directly used for steady-state kinetic analysis.

In cell or tissue extracts, this method monitors the combined activity of all active SUMO proteases in a given sample (and potentially additional, less specific proteases as well). When combined with knockout or knockdown of individual proteases, one may be able to deduce the contribution of a distinct protease to the observed cleavage activity.

The following procedure is designed to measure SUMO protease activity in lysates from mouse heart tissue or HeLa cells, but the assay can in principle be applied to any other kind of cell or tissue. Fig. 2 gives an example of SUMO protease activity toward SUMO2-AMC in HeLa cell lysates derived from cells under control conditions or cells that were exposed to heat stress. When compared to control conditions heat stress reduces the cellular activity of SUMO proteases. A sample in which N-ethylmaleimide (NEM) was added to the lysis buffer serves as a negative control because alkylation of cysteine residues by NEM will inhibit SUMO protease activity.

### 2.1.1 Equipment

- Standard cell-culture equipment
- Cell scraper
- Bench top microcentrifuge for Eppendorf tubes
- Centrifuge with rotor for plate centrifugation
- Sonicator (Sonics Vibra-cell VCX 130)
- 384-Well plate, black, flat bottom
- Plate reader (BioTek Synergy H1)
- Ice bucket



**Fig. 2** Monitoring the impact of heat stress on SUMO protease activity in HeLa cells by SUMO2-AMC assay. HeLa cells were incubated under standard conditions at 37°C or exposed to heat stress (43°C for 30 min) as indicated. Cell lysates were prepared as described in the protocol under [Section 2.1.3.1](#). For a negative control, NEM was added to the lysis buffer. 10 µg cell lysate were incubated with 500 nM SUMO2-AMC and fluorescence was followed for 30 min. Measurements were performed in duplicate.

- –80°C freezer
- Liquid nitrogen
- Tissue dounce homogenizer (Wheaton 357538 glass 1 mL, with 2 (loose and tight) pestles)

### 2.1.2 Buffers and reagents

- Cell line (e.g., HeLa) and appropriate cell-culture medium.
- SUMO1-AMC or SUMO2-AMC substrate (BostonBiochem or LifeSensors).
- Kit to determine protein concentration (e.g., DC protein assay, Bio-Rad).
- Phosphate-buffered saline (PBS).
- 1 M NEM stock solution in DMSO.
- 1 M DTT stock solution in H<sub>2</sub>O.
- Protease inhibitor cocktail (Complete, Roche) or individual inhibitors: Aprotinin (10 mg/mL in H<sub>2</sub>O), Leupeptin (10 mg/mL in H<sub>2</sub>O), Pepstatin A (1 mg/mL in DMSO), and PMSF (0.2 M in isopropanol).

- SEM buffer: 0.25 M sucrose, 20 mM MOPS-KOH (pH 7.4), 1 mM EDTA-NaOH pH 8). Store at 4°C. Immediately before use, supplement SEM buffer with Aprotinin (use at 1/5000), Leupeptin (1/5000), Pepstatin A (1/1000), PMSF (1/200), DTT (1/1000) and, for the negative control, add NEM (to 10 mM).
- Activity assay buffer: 50 mM Tris-HCl (pH 7.5), 0.1 mg/mL BSA, 10 mM DTT.

### 2.1.3 Procedure

#### 2.1.3.1 Lysis of mammalian cells

- Prepare 3 × 10 cm dishes of HeLa cells grown to reach confluence on the day of the experiment.
- Incubate one dish at 43°C (heat-shock sample) for 30–60 min.
- The remaining dishes are kept at 37°C. One will serve as the 37°C control, while the other will be used as the negative control (lysis in NEM-containing buffer).
- Prior to lysis, supplement lysis buffer with protease inhibitors and DTT.
- Add NEM to a final concentration of 10 mM to the lysis buffer that will be used for the negative control sample (see Note “a” in [Section 2.1.4](#)).
- After incubation at either 37°C or 43°C, wash cells 3 × with PBS.
- Remove PBS and scrape cells in 1 mL of SEM lysis buffer on ice, then transfer the lysate into microcentrifuge tubes.
- From this step forward, keep cell lysates on ice.
- Briefly sonicate cell lysates to dissociate aggregates.
- Determine protein content of lysates and adjust samples with SEM buffer to the same protein concentration (e.g., 1 mg/mL) (see Note “b” in [Section 2.1.4](#)).
- For storage: make aliquots and snap-freeze in liquid nitrogen and store at –80°C.

#### 2.1.3.2 Lysis of mouse heart tissue

- Quickly excise the heart from an euthanized mouse (see Note “c” in [Section 2.1.4](#)).
- Determine the wet weight of the heart and snap-freeze immediately in liquid nitrogen (see Note “d” in [Section 2.1.4](#)).
- Homogenize the tissue in 10 × volume SEM buffer (e.g., 100 mg tissue in 1 mL SEM buffer supplemented with DTT and protease inhibitors, ±NEM) at 4°C with a tissue douncer.

- Transfer the homogenate into a 1.5 mL Eppendorf tube and centrifuge with a bench top centrifuge at  $18,000 \times g$  and  $4^{\circ}\text{C}$  for 10 min.
- Transfer the supernatant into a new 1.5 mL Eppendorf tube and discard the pellet.
- Determine protein concentration according to manufacturer's instructions (see Note "b" in [Section 2.1.4](#)).
- Use a fresh aliquot of the lysate for AMC assay. Alternatively, snap-freeze the lysates in appropriate aliquots in liquid nitrogen and store them in  $-80^{\circ}\text{C}$  until use.

#### 2.1.3.3 SUMO-AMC measurement

- Set up program for the plate reader:  $\lambda_{\text{Ex}} = 380 \text{ nm}$ ,  $\lambda_{\text{Em}} = 460 \text{ nm}$  (see Note "e" in [Section 2.1.4](#)).
- Dispense cell lysates (corresponding to 5–30  $\mu\text{g}$  total protein) into the 384-well plate on ice (see Notes "f" and "g" in [Section 2.1.4](#)).
- For blank, include one well where lysate is replaced by the corresponding volume of SEM buffer.
- Prepare assay master-mix: for one reaction use 500 nM SUMO1-AMC or SUMO2-AMC substrate and adjust with activity assay buffer to a total volume of 50  $\mu\text{L}$  (including volume of lysate).
- Add appropriate amount of this master-mix to cell lysate in the plate (e.g., mix 10  $\mu\text{L}$  of 1 mg/mL HeLa cell lysate with 40  $\mu\text{L}$  master-mix; final volume: 50  $\mu\text{L}$ ).
- Quickly spin down the plate to collect liquids at the bottom of the plate and immediately start measurement.

#### 2.1.4 Notes

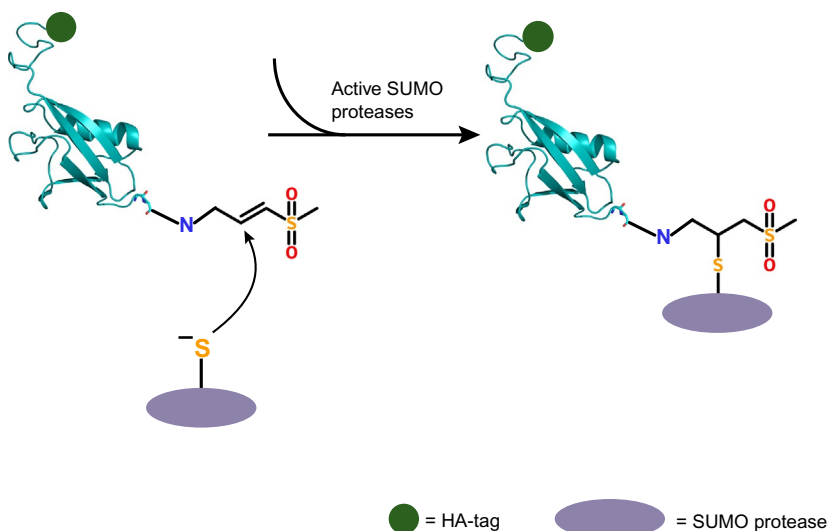
- a It is advisable to prepare ready-to-use lysis buffers during incubation time so that cells can be lysed rapidly.
- b Use an assay for protein quantification compatible with 0.25 M sucrose, 20 mM MOPS, 1 mM EDTA, and 1 mM DTT.
- c Experiments involving animals must be carried out in accordance with all appropriate national and institutional regulations.
- d Tissue samples can be stored in cryotubes at  $-80^{\circ}\text{C}$  for up to 1 year.
- e It is crucial to start the measurement immediately after lysate and AMC substrate have been mixed, therefore the plate reader settings should be adjusted in advance.
- f Amount of cell lysate may vary depending on cell line or tissue as well as SUMO1- or SUMO2-AMC. Take at least 30  $\mu\text{g}$  protein from

mouse heart tissue for SUMO1-AMC assay and 15 µg for SUMO2-AMC measurements.

**g** Measurements should be performed at least in duplicates.

## 2.2 Using SUMO-VS probes to monitor SUMO protease activities in cell and tissue lysates

To determine the activity and paralog specificity of distinct SUMO proteases, irreversible active site binders of SENPs have been developed (Borodovsky et al., 2005; Hemelaar et al., 2004; Kolli et al., 2010). In the commercially available probes, a vinyl sulfone (VS) group is covalently linked to the GlyGly motif at the C-terminus of mature SUMO isoforms (SUMO1-VS or SUMO2-VS). The VS-group reacts with the catalytic cysteine of Cys proteases by forming a noncleavable thioether bond. In this reaction, the sulfur of the catalytic cysteine attacks the double bond of the vinyl group and forms a covalent bond (Reverter & Lima, 2009) (Fig. 3). When linked to SUMO, the VS group therefore functions as a specific suicide trap for SUMO proteases. Commercially available SUMO-VS probes



**Fig. 3** Schematic view of the SUMO-VS probes. In the commercially available probes, a vinyl sulfone (VS) group is covalently linked to the GlyGly motif at the C-terminus of mature SUMO isoforms (SUMO1-VS or SUMO2-VS). The VS-group reacts with the catalytic cysteine of Cys proteases by forming a noncleavable thioether bond. In this reaction, the sulfur of the catalytic cysteine attacks the double bond of the vinyl group and forms a covalent bond. In addition, an HA-tag is bound to the N-terminus of SUMO.

harbor an HA-tag at the N-terminus of SUMO to allow detection of SUMO-VS-protease adducts after SDS-PAGE separation by anti-HA immunoblotting. In HeLa cell extracts, distinct anti-HA reactive bands at 180, 95, and 75 kDa, which mainly correspond to SENP6, SENP1, and SENP3 adducts and are sensitive to NEM treatment, are typically detected.

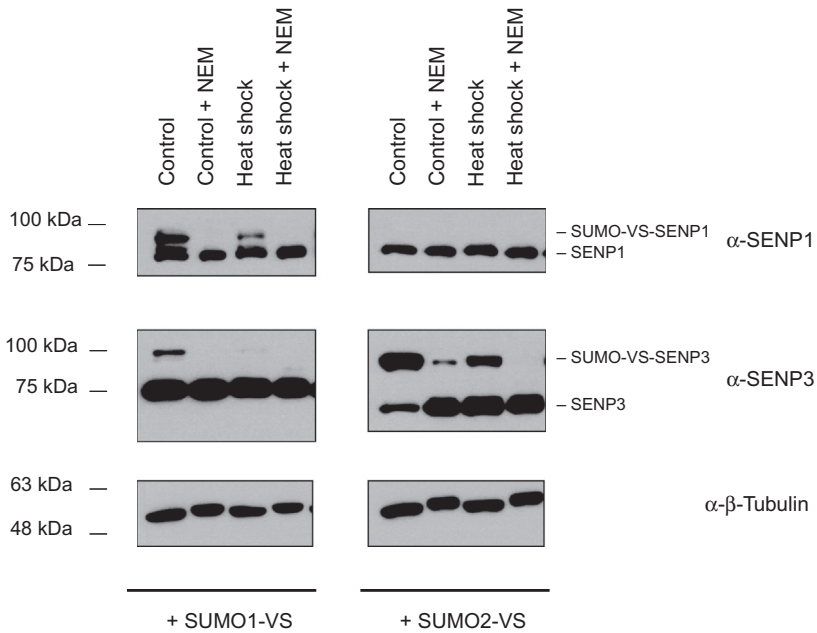
Distinct SUMO-VS-SENP adducts can be detected by antibodies directed against specific SENP family members. By adding HA-SUMO1-VS or HA-SUMO2-VS to cell extracts and following the conversion of free SENP to the SUMO-VS-SENP adduct over time, the activity of a distinct SENP under varying experimental conditions can be monitored. By comparing adduct formation with SUMO1-VS vs SUMO2-VS, the preference for specific SUMO proteases toward either SUMO1 or SUMO2/3 can be examined. Typical results obtained with SENP1 and SENP3 in HeLa cells under normal conditions or upon heat stress are shown in Fig. 4. In accordance with published work, activity of both SENP1 and SENP3 is reduced upon heat stress (Mendes, Grou, Azevedo, & Pinto, 2016; Mulder et al., 2018). Fig. 5 shows immunoblot analyses with both an anti-HA antibody, which shows the distribution of SUMO-VS-reactive proteins in mouse heart lysates, and an anti-SENP3 antibody, highlighting SENP3 reactivity in the same tissue.

### 2.2.1 Equipment

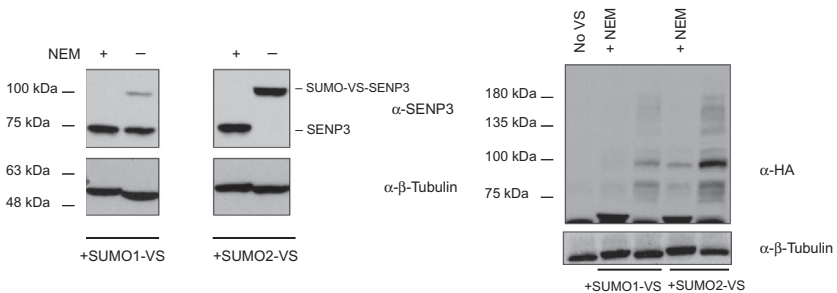
- Standard cell-culture equipment
- Bench top microcentrifuge for Eppendorf tubes
- Sonicator (Sonics Vibra-cell VCX 130)
- Ice bucket
- $-80^{\circ}\text{C}$  freezer
- Liquid nitrogen
- Tissue douncer (Wheaton)
- Thermomixer (Eppendorf Thermomixer compact, suitable for 1.5 mL tubes)
- Standard equipment for SDS-PAGE and immunoblotting

### 2.2.2 Buffers and reagents

- Cell line (e.g., HeLa) and appropriate cell-culture medium.
- HA-SUMO1-VS or HA-SUMO2-VS substrate (e.g., BostonBiochem).
- Kit for protein quantification (e.g., DC protein assay, Bio-Rad).
- PBS.
- 1 M NEM stock solution in DMSO.



**Fig. 4** Monitoring the impact of heat stress on activity of distinct SUMO proteases in HeLa cells by SUMO-VS modification assays. HeLa cells were incubated under standard conditions at 37°C or exposed to heat stress (43°C for 30 min) as indicated. Cell lysates were prepared as described under [Section 2.1.3.1](#). For a negative control, NEM was added to the lysis buffer. Lysates were incubated with HA-SUMO1-VS or HA-SUMO2-VS for 15 min at 25°C. Immunoblotting was performed with anti-SENP1 and SENP3 antibodies, respectively. SUMO-VS-SENP adduct formation is a measure of SENP activity and the ratio of free SENP to the SUMO-VS adduct reflects SENP activity. The example shows the preference of SENP1 and SENP3 toward SUMO1-VS and SUMO2-VS, respectively. Decreased SENP activity under heat stress is evident by the reduced levels of SUMO-VS adducts. Addition of NEM largely reduces SENP activity.



**Fig. 5** Monitoring activity of distinct SUMO proteases in heart tissue lysates by SUMO-VS assays. Mouse heart tissue was lysed as described in the protocol under [Section 2.1.3.2](#). Lysate (corresponding to 40 µg total protein) was incubated with HA-SUMO1-VS or HA-SUMO2-VS, separated by SDS-PAGE and immunoblot analysis was done using antibodies against SENP3, HA, and Tubulin. SENP3 preferentially reacts with SUMO2-VS as indicated by its almost complete conversion to the SUMO2-VS-SENP3 adduct. NEM completely blocks this reaction. Immunoblotting against the HA-tag of either HA-SUMO1-VS or HA-SUMO2-VS shows several different species of active SUMO proteases in mouse heart tissue that predominantly react with HA-SUMO2-VS.

- 1 M DTT stock solution in H<sub>2</sub>O.
- Protease inhibitor cocktail or individual inhibitors: Aprotinin (10 mg/mL in H<sub>2</sub>O), Leupeptin (10 mg/mL in H<sub>2</sub>O), Pepstatin A (1 mg/mL in DMSO), and PMSF (0.2 M in isopropanol).
- SEM buffer: 0.25 M sucrose, 20 mM MOPS-KOH (pH 7.4), 1 mM EDTA-NaOH pH 8). Store at 4°C. Freshly supply SEM buffer before use with Aprotinin (use 1/5000), Leupeptin (use 1/5000), Pepstatin A (use 1/1000), PMSF (use 1/200), DTT (use 1/1000), and only for the negative control add NEM (10 mM).
- Laemmli buffer.
- Standard SDS-PAGE reagents and Bisacrylamide gels (percentage suitable to separate desired SENPs by size).
- Antibodies against SENPs, HA-tag, and tubulin (loading control).

### 2.2.3 Procedure

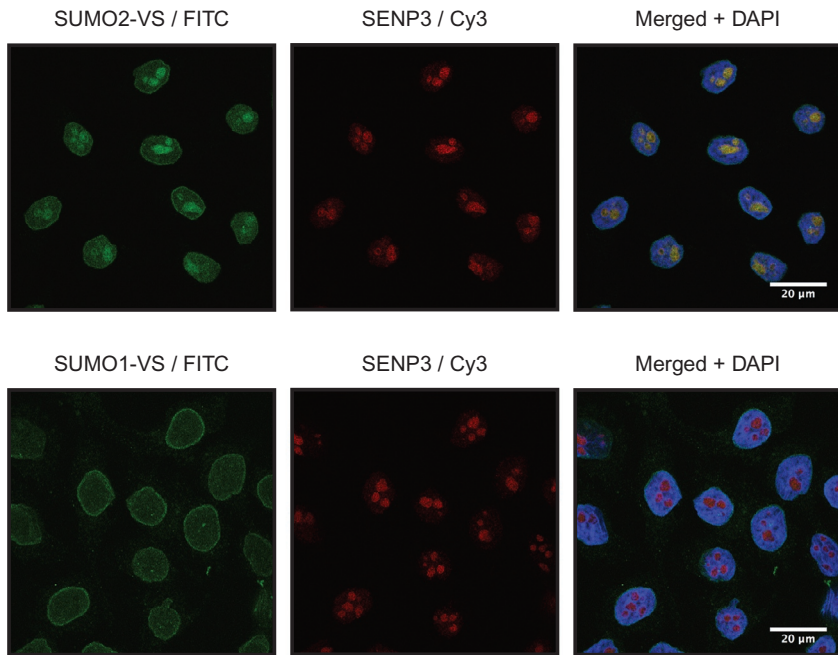
- Prepare cell or tissue lysate in SEM buffer ± NEM as described for AMC assay.
- Determine protein content and incubate 100 µg lysate with 50 ng HA-SUMO-VS (either SUMO1-VS or SUMO2-VS) for 15 min in a thermomixer at 25°C.
- Stop reaction with Laemmli buffer and heat for 5 min to 95°C.
- Separate an aliquot of the sample by SDS-PAGE and perform immunoblot using anti-HA antibody or antibody directed against a specific SUMO protease.

### 2.2.4 Notes

For best results, add HA-SUMO-VS probes to freshly isolated lysates. One can label, e.g., 100 µg cell lysates or mouse heart proteins (by adding 50 ng HA-SUMO-VS probe) and use the samples for several Western blots. In the case of mouse heart tissue, load 30–40 µg protein onto gel for subsequent Western blotting with anti-SENP or anti-HA antibodies.

## 2.3 Using SUMO-VS probes for in situ analysis of SUMO protease activity in mammalian cells

HA-SUMO-VS probes can also be used to monitor SUMO protease activity in situ by immunofluorescence microscopy. This aspect is particularly important since SUMO proteases are compartmentalized in distinct subcellular regions. Entry of the HA-SUMO-VS probe into cells requires their permeabilization prior to fixation. Subsequently, a standard



**Fig. 6** In situ analysis of SENP activity by HA-SUMO-VS immunofluorescence in HeLa cells. After permeabilization with Triton-X-100, HeLa cells were incubated with either HA-SUMO1-VS or HA-SUMO2-VS probes and then fixed with PFA. Standard co-immunofluorescence analysis was carried out by using anti-HA/FITC in parallel with anti-SENP3/Cy3-staining; DAPI is used to stain DNA. The predominantly nucleolar HA-SUMO2-VS signal in HeLa cells colocalizes with SENP3, whereas the HA-SUMO1-VS signal is concentrated mainly in the nucleoplasm. Additionally, both probes exhibit nuclear rim staining. Scale bar = 20  $\mu\text{m}$ .

immunofluorescence protocol can be performed. This includes fixation of cells, staining with primary antibody directed against the HA-tag followed by fluorescently labeled secondary antibodies and subsequent confocal microscopy (Fig. 6).

### 2.3.1 Equipment

- Standard cell-culture equipment
- Cell counter
- 12-well tissue culture plates
- 18mm cover slips
- Forceps
- Dark box

- Whatman paper
- Slides
- Confocal microscope

### **2.3.2 Buffers and reagents**

- Mammalian cell line (e.g., HeLa) including appropriate medium
- HA-SUMO1-VS or HA-SUMO2-VS substrate (e.g., BostonBiochem)
- PBS
- Triton-X-100
- 4% Paraformaldehyde (PFA) in PBS (Morphisto)
- BSA
- Tween 20
- PBS-T (PBS + 0.1% Tween 20)
- Primary antibodies against SENPs and HA-tag
- Fluorophore-coupled secondary antibodies
- DAPI 1 mg/mL stock (in PBS)
- ProLong Gold antifade mounting medium (Thermo Fisher)

### **2.3.3 Procedure**

- Seed  $5 \times 10^4$  HeLa cells on cover slips in 1 mL cell-culture medium per well on a 12-well plate and cultivate overnight at 37°C.
- Remove medium and wash cells  $2 \times$  in PBS.
- Permeabilize cells in 0.2% Triton X/PBS for 5 min (see Note “a” in [Section 2.3.4](#)).
- Carefully remove permeabilization solution and wash cells  $1 \times$  gently in PBS.
- Remove PBS wash and add 50–100 ng HA-SUMO1-VS or HA-SUMO2-VS substrate in 50  $\mu$ L PBS to cover slips and incubate 10 min at room temperature.
- Wash cells  $3 \times$  gently with PBS.
- Fix cells in 4% PFA in PBS for 15 min at room temperature.
- Wash cells  $3 \times$  in PBS.
- Block nonspecific binding sites in cells using 2% BSA in PBS-T for 20 min (see Note “b” in [Section 2.3.4](#)).
- Wash cells  $1 \times$  in PBS.
- Add primary antibodies against HA tag (e.g., mouse anti-HA from Covance 1:250) and against SENP (e.g., rabbit anti-SENP3 from cell signaling 1:700) in 50  $\mu$ L of 2% BSA/PBS-T and incubate for 30 min at room temperature.

- Wash cells  $3 \times$  with PBS.
- Add fluorophore-coupled secondary antibodies (e.g., FITC-coupled anti-mouse 1:500 and Cy3-coupled antirabbit 1:500) in  $50 \mu\text{L}$  2% BSA/PBS-T and incubate 30 min in the dark.
- Add DAPI 1:1000 in PBS ( $1 \mu\text{g}/\text{mL}$  final cc.) 1 min.
- Wash cells  $3 \times$  with PBS.
- Wash cells  $2 \times$  with  $\text{ddH}_2\text{O}$  and remove excess liquid from coverslip.
- Place coverslip sample-side down onto ProLong Gold reagent on the glass slide.
- Keep slides in dark at  $4^\circ\text{C}$  until confocal microscopy is done.

### 2.3.4 Notes

- a As long as permeabilized cells are not fixed they detach easily from cover slips. All washing steps should therefore be performed carefully. In order to minimize cell loss an alternative detergent that can be used for permeabilization is saponin (0.1% saponin in PBS + 5 mM  $\text{MgCl}_2$ ). Since permeabilization by saponin is not permanent, one has to incubate not only the SUMO-VS substrates but also both primary and secondary antibodies in saponin-containing solution. As an alternative to detergent permeabilization, electroporation has been used to bring the probes into cells (Mulder et al., 2018).
- b If a low signal-to-noise ratio is obtained, the concentration of BSA can be increased from 2% to 5% in the blocking as well as antibody incubation solutions.



## 3. Defining substrate specificity of distinct SUMO proteases

Defining the substrate specificity of a given SENP toward ectopically expressed or endogenous substrates in mammalian cells is still a major challenge. Many studies have relied on ectopic expression of SUMO proteases to demonstrate their function in a distinct deconjugation process. Because overexpression of SUMO proteases/isopeptidases may lead to loss of specificity, a more reliable approach is to monitor the SUMOylation status of the protein of interest upon depletion (by knockdown or knockout) of a specific SENP. Since typically only a small fraction of a given SUMO target is found in the modified form, detection of SUMO conjugates requires their enrichment from cell lysates. This can either be done by using anti-SUMO1 or anti-SUMO2 antibodies. A more convenient and

straightforward way is the enrichment of SUMO conjugates on Ni-NTA beads from cells expressing His-SUMO forms (Tatham, Rodriguez, Xirodimas, & Hay, 2009). The Ni-NTA-polyhistidine interaction tolerates denaturing conditions, which is crucial for the inactivation of endogenous SUMO-specific isopeptidases during purification. While transient expression of His-SUMO forms is an option, the use of stable cell lines expressing His-SUMO1 or His-SUMO2 at levels comparable to endogenous SUMO is highly suggested. We use His-SUMO expressing cell lines generated by the T-REx Flp-In™ system. These cell lines harbor a single integration of the gene of interest, which is under control of a tetracycline-regulated cytomegalovirus (CMV) promoter. By denaturing Ni-NTA pulldown, SUMOylated versions of the protein of interest can be enriched in control cells, and depletion of a given SENP will allow the effects on SUMO conjugates to be measured by Western blotting.

### 3.1 Equipment

- Standard cell-culture equipment
- His-SUMO1- or His-SUMO2-expressing cell line (expressed from a tetracycline-inducible promoter) (see Note “a” in Section 3.4)
- Cell scraper
- Thermomixer
- Bench top centrifuge including cooling function
- Vortexer
- Ice bucket
- Vacuum concentrator (Speedvac) (see Note “b” in Section 3.4)
- Magnetic Ni-NTA-beads from Qiagen
- Rotating wheel
- Standard Western blot equipment
- Standard SDS-PAGE reagents and Bisacrylamide gels

### 3.2 Buffers and reagents

- Doxycycline (final concentration 1 µg/mL)
- siRNA directed against SUMO-specific isopeptidase gene (MGW Eurofins genomics)
- Lipofectamine RNAi MAX (Invitrogen)
- Plasmid DNA for wild-type BHLHE40 or SUMOylation-deficient mutant BHLHE40<sup>K159R, K279R</sup>
- FuGENE transfection reagent (Promega)

- Opti-MEM
- PBS
- Lysis buffer (6 M guanidine-HCl, 0.1 M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.1 M Tris, 0.05% Tween, pH 8)
- Wash buffer A (8 M urea, 0.1 M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.01 M Tris, 0.05% Tween, pH 8)
- Wash buffer B (8 M urea, 0.1 M  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.01 M Tris, 0.05% Tween, pH 6.4)
- 50% TCA (trichloroacetic acid)
- Ethanol, 100%, ice cold
- Laemmli gel loading buffer

### 3.3 Procedure

- On day 1, seed 10-cm dishes with cells that have an integrated His-SUMO1 or His-SUMO2 gene; use one dish per condition and do reverse transfection with siRNA against desired SUMO isopeptidase according to manufacturer's instructions. Cultures are grown at 37°C (see Note "c" in [Section 3.4](#)).
- The day after, transfect cells with plasmid DNA for either wild-type BHLHE40 or SUMOylation-deficient mutant BHLHE40<sup>K159R,K279R</sup>. Therefore, mix 7.5 µg plasmid DNA with 25 µL FuGENE in 500 µL Opti-MEM and incubate this mix for 25 min at room temperature, then pipet drop-wise to the cells.
- On day 3, induce expression of His-SUMO1 or His-SUMO2 by addition of doxycycline and incubate overnight.
- On day 4, harvest cells and perform His-Pulldown as described in the following steps (see Note "d" in [Section 3.4](#)).
- Wash cells twice in PBS and scrape them off in 1 mL of lysis buffer per 10 cm dish.
- Transfer lysed cell suspension to microcentrifuge tubes and heat at 99°C for 15 min.
- Centrifuge for 20 min at  $18,000 \times g$  at room temperature.
- Transfer the supernatant into new Eppendorf tube.
- Take 100 µL of the supernatant and use for TCA precipitation (see Note "e" in [Section 3.4](#)): Add 20 µL of 50% TCA, vortex and incubate on ice for 15 min.
- Centrifuge for 15 min at  $18,000 \times g$  at room temperature.
- Discard supernatant and dissolve pellet in 200 µL ice cold ethanol (100%), vortex.

- Centrifuge for 20 min at  $18,000 \times g$  at room temperature.
- Carefully remove supernatant and dry the pellet for 10 min at  $42^{\circ}\text{C}$  in a Speedvac (see Note “b” in [Section 3.4](#)).
- Dissolve pellet in 200  $\mu\text{L}$  Laemmli buffer.
- Heat to  $95^{\circ}\text{C}$  for 10 min. “Input material” sample is ready for Western blot analysis or can be stored at  $-20^{\circ}\text{C}$ .
- For pulldown: Incubate the remaining lysate with 30  $\mu\text{L}$  Ni-NTA beads per 10 cm dish.
- Rotate overnight at room temperature on a rotating wheel.
- On the next day, discard the supernatant and wash beads as follows:  $3 \times$  with 1 mL buffer A,  $2 \times$  with 1 mL buffer B, and a final wash step in PBS.
- Add 30  $\mu\text{L}$  Laemmli buffer to beads and heat for 10 min to  $95^{\circ}\text{C}$ . “Pulldown” samples are ready for Western blot analysis or storage at  $-20^{\circ}\text{C}$ .
- Western blot analysis of SUMOylated proteins: Check for efficient extraction and pulldown by blotting with an antibody against the His-tag using both input and pulldown samples (see Note “f” in [Section 3.4](#)). Remaining pulldown sample is used for immunoblotting against the protein of interest in its potentially SUMOylated form.
- Also verify the efficiency of SUMO protease knockdown by immunoblot analysis using an antibody to the examined protease (not shown in [Fig. 7](#)).

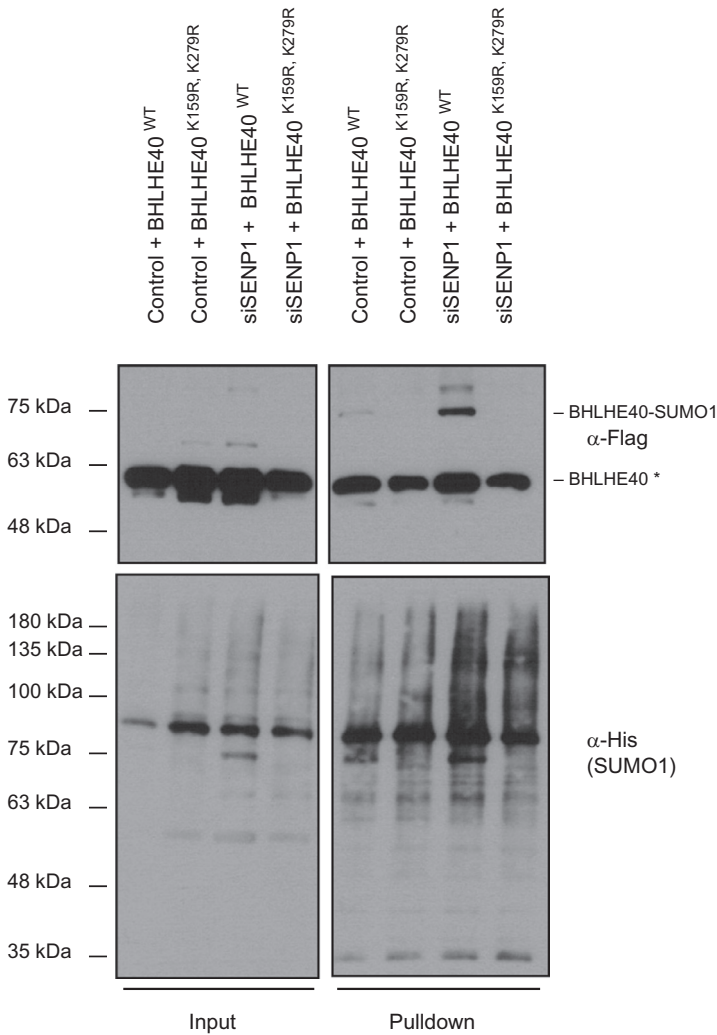
### 3.4 Notes

- a** If a His-SUMO-expressing cell line is not available, cells can also be transiently transfected with plasmids expressing His-SUMO1 or His-SUMO2.
- b** If no vacuum concentrator is available, precipitated lysate can also be air dried over night at room temperature.
- c** As a control, a condition without doxycycline induction can be included or in case of transient transfection, an HA-SUMO construct can be used.
- d** All steps are performed at room temperature.
- e** This will later be used as input material.
- f** Only use 1–2  $\mu\text{L}$  of pulldown samples to check for pulldown efficiency.



## 4. Summary and conclusion

In this chapter, we describe convenient assays for monitoring SUMO protease activity with the help of commercially available activity-based



**Fig. 7** Identification of SUMOylated BHLHE40 as a SENP1 target in cells. HeLa cells stably expressing His-SUMO1 were transfected with siRNA directed against SENP1 or a control siRNA. The day after, wild-type BHLHE40 or SUMOylation-deficient mutant BHLHE40<sup>K159R, K279R</sup> was expressed. After denaturing cell lysis and Ni-NTA pulldown, input and pulldown samples were immunoblotted against Flag-BHLHE40 and His-SUMO. The SUMOylated version of BHLHE40 is weakly detectable in control cells but strongly induced after SENP1 knockdown in the wild-type BHLHE40 pulldown sample, but not the BHLHE40<sup>K159R, K279R</sup> sample. Asterisk denotes unmodified BHLHE40 in the Ni-NTA pulldown. Adapted from Kunz, K., Wagner, K., Mendler, L., Holper, S., Dehne, N., & Muller, S. (2016). SUMO signaling by hypoxic inactivation of SUMO-specific isopeptidases. *Cell Report*, 16(11), 3075–3086. doi: 10.1016/j.celrep.2016.08.031.

SUMO substrates and probes. Recently the Ovaa lab has expanded the toolbox of activity-based SUMO probes by optimizing the chemical synthesis of full-length SUMO paralogs (Mulder et al., 2018). An important advance of their work was the development of diSUMO probes that can be used to monitor SUMO protease activity in cleaving SUMO linkages. Given that SUMO chain function is still not well understood these reagents will be of great importance in the field (Gartner et al., 2018). Generally, many publications have focused on alterations in SUMO protease levels under pathological conditions, including cancer. However, measuring activity rather than levels is a more reliable way to determine the impact of distinct SENP family members on cell physiology.

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