



Evaluation of the activity and substrate specificity of the human SENP family of SUMO proteases

Andreia V. Mendes¹, Cláudia P. Grou¹, Jorge E. Azevedo², Manuel P. Pinto³

Originally published in *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research*. 2016 Jan; 1863: 139-147, Elsevier. DOI: 10.1016/j.bbamcr.2015.10.020

¹Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal; Organelle Biogenesis and Function Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal.

²Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal; Organelle Biogenesis and Function Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal; Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal. Electronic address: jazevedo@ibmc.up.pt.

³Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal; Organelle Biogenesis and Function Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Electronic address: mabp@ibmc.up.pt.

ABSTRACT

Protein modification with the small ubiquitin-like modifier (SUMO) is a reversible process regulating many central biological pathways. The reversibility of SUMOylation is ensured by SUMO proteases many of which belong to the sentrin/SUMO-specific protease (SENP) family. In recent years, many advances have been made in allocating SENPs to specific biological pathways. However, due to difficulties in obtaining recombinant full-length active SENPs for thorough enzymatic characterization, our knowledge on these proteases is still limited. In this work, we used in vitro synthesized full-length human SENPs to perform a side-by-side comparison of their activities and substrate specificities. ProSUMO_{1/2/3}, RanGAP₁-SUMO_{1/2/3} and polySUMO_{2/3} chains were used as substrates in these analyses. We found that SENP₁ is by far the most versatile and active SENP whereas SENP₃ stands out as the least active of these enzymes. Finally, a comparison between the activities of full-length SENPs and their catalytic domains suggests that in some cases their non-catalytic regions influence their activity.

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

Version: Postprint (identical content as published paper) This is a self-archived document from i3S – Instituto de Investigação e Inovação em Saúde in the University of Porto Open Repository For Open Access to more of our publications, please visit <http://repositorio-aberto.up.pt/>

INTRODUCTION

The post-translational modification of proteins with the small ubiquitin-like modifier (SUMO) plays a central role in cell homeostasis by regulating a large number of key biological processes such as DNA repair, transcription and cell cycle progression [1], [2]. Mammals express three SUMO paralogues—SUMO₁, and the nearly identical and functionally indistinguishable SUMO₂ and SUMO₃ [3], [4], [5], [6], [7]. The three SUMO paralogues are conjugated to target-proteins through an enzymatic cascade comprising one SUMO-activating enzyme [8], one SUMO-conjugating enzyme [9] and, in some cases, also a SUMO ligase [10]. This machinery activates the C-terminal glycine carboxyl group of SUMO in an ATP-dependent process and conjugates it to the ϵ -amino group of a lysine residue in target proteins [2], [11]. As with ubiquitination, proteins can be modified with a single SUMO molecule (monoSUMOylation) or with a chain of SUMO molecules (polySUMOylation) [2]. Polymeric chains are built by attaching a new SUMO moiety to lysine 11 of the previous SUMO molecule [6], [12]. Due to the fact that SUMO₁ lacks this lysine residue, only SUMO₂ and SUMO₃ are involved in polySUMO chain elongation [12].

Protein modification by SUMO is a highly dynamic and transient process, readily reverted by the action of SUMO proteases, hydrolases that remove SUMO or SUMO chains from SUMOylated proteins [2], [13]. Most SUMO proteases belong to the so-called sentrin/SUMO-specific protease (SENP) family of cysteine proteases. In mammals there are six SENPs. They are often classified into three groups according to sequence relationships and predominant subcellular localization [2], [13], [14], [15]. The first group comprises SENP1 and SENP2, both localizing mainly to the nuclear periphery [16], [17], [18]. The second group consists of SENP3 and SENP5 [19], [20], which localize mostly to the nucleolus. The third group comprises SENP6 and SENP7 [21], [22], two enzymes mainly found in the nucleoplasm. All SENPs are structurally organized into a C-terminal catalytic domain and a non-conserved N-terminal region which comprises around two-thirds of their polypeptide chains [2], [13].

In addition to SENPs, three other SUMO proteases have been described recently in mammals: the deSUMOylating isopeptidases DeSI-1 and DeSI-2 [23], and the ubiquitin-specific protease-like 1 (USPL1) [24]. These enzymes are also cysteine proteases but they are very distantly related to the SENP family. Also, they probably act on a very restricted set of substrates because knockdown of any of these enzymes in cell lines does not lead to detectable alterations in the bulk of SUMO conjugated products [23], [24]. The minor roles of each of these enzymes in the global cell SUMOylation status [23], [24] contrast with the large accumulations of SUMOylated proteins detected in cells lacking any of the SENP family members [19], [22], [25], [26], [27], [28], [29].

Besides a major role in controlling the SUMOylation status of many proteins, SENPs are also involved in the de novo synthesis of SUMO [19], [20], [30], [31], [32]. Indeed, the three SUMO paralogues are expressed as precursor proteins (proSUMO_{1/2/3}) that must be processed to yield the active/conjugatable proteins. Processing involves the removal of small peptide extensions that are present at the C termini of proSUMOs [2].

Although the involvement of SENPs in several biological processes is now well established, their substrates specificities and activities are still ill-characterized. A major problem in addressing this issue stems from the fact that, thus far, it has not been possible to obtain recombinant active full-length SENPs for thorough enzymatic characterization. Thus, most studies on SENP activity and

specificity have used recombinant truncated proteins comprising only their catalytic domains in enzymatic assays with natural or artificial substrates [19], [22], [30], [33], [34], [35], [36], [37]. In the case of SENP₃, even this type of data are lacking due to problems in obtaining a stable and active catalytic domain [30]. Other studies have looked at the reactivity of endogenous or epitope-tagged SENPs in cell extracts towards activity-based irreversible inhibitors such as SUMO₁ and SUMO₂ vinyl sulfones (VS) [21], [38], [39]. One of these studies revealed that (full-length) SENP₁ reacts equally well with both SUMO₁-VS and SUMO₂-VS whereas all the other SENPs show a preference for SUMO₂-VS [38]. Interestingly, it was also shown that the recombinant catalytic domains of SENPs no longer display a SUMO paralogue preference when tested with the activity-based inhibitors. Seemingly, the N-terminal non-catalytic domains of SENPs somehow modulate SENP activity/substrate specificity [2], [13], thus raising some doubts on whether or not the enzymatic data reported for SENP catalytic domains can be extrapolated to the full-length enzymes. Clearly, additional studies using intact enzymes and their natural substrates are needed to better understand the properties of these proteases.

Materials and Methods

Plasmids and proteins

The cDNAs encoding human full-length SENP_{1/2/5/6} were optimized for *Escherichia coli* expression (GenScript) and cloned into the NdeI/XhoI sites of pET28a. The plasmids encoding human full-length SENP₃ and SENP₇ in pET28a were described before [40]. Flag-tagged pcDNA5/FRT/TO-derived constructs of wild type (WT) SENP₇, of a mutant lacking seven putative SUMO-interacting motifs (SIMs) in the N-terminal region (SM) and of the catalytic inactive C992A mutant (CA) were kindly provided by Dr. Joanna R. Morris (University of Birmingham, UK) [41]. These plasmids were digested with KpnI/XhoI and the DNA inserts were cloned into pcDNA3.1, which contains a T7 promoter, for *in vitro* synthesis. The pET28a-derived plasmids coding for the catalytic domains of human SENP_{1/2/5-7} were a kind gift from Dr. Guy Salvesen [30]. The cDNA encoding SENP₃ catalytic domain, SENP₃(307–574), was amplified by PCR using as template SENP₃-pET28a and cloned into the NdeI/XhoI sites of pET28a. A plasmid encoding untagged human full-length RanGAP₁ (RanGAP₁-pET23a) was obtained from Addgene (plasmid #13387) [42]. The plasmid ΔC1-PEX5-pET28a was described elsewhere [43].

Recombinant full-length His₆-SENP₁ was expressed in Rosetta 2(DE3) *E. coli* cells with 0.5 mM IPTG for 3 h at 30 °C. After cell lysis in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (w/v) glycerol) supplemented with 1:500 (v/v) protease inhibitors cocktail (Sigma), 0.25 mg/ml PMSF and 1 mM DTT, inclusion bodies were washed twice with the same buffer in the presence of 1% (w/v) Triton X-100. The plasmid pTYB-SUMO₂ [40] was used to produce HA-tagged SUMO₂ vinyl methyl ester (HA-SUMO₂-VME) as previously described [44], excluding the ion-exchange purification step. Plasmids encoding SUMO₂, SUMO₃, Ubc9 and SAE₁/SAE₂ (SUMO E1) were kindly provided by Dr. Frauke Melchior [45], [46] and used to prepare the respective recombinant proteins as described [47]. Recombinant untagged SUMO₁, proSUMO₁₋₃, polySUMO₂ and polySUMO₃ were purchased from Boston Biochem or Enzo Life Sciences. Radiolabeled proteins were synthesized *in vitro* using pET- or pcDNA3.1-based plasmids with the TNT[®] T7 Quick Coupled Transcription/Translation System (Promega), in the presence of EasyTag[™] L-[³⁵S]-Methionine (Perkin Elmer, specific activity > 1000 Ci (37.0 TBq)/mmol), according to the manufacturer's instructions. Translation reactions were stopped by incubation with 1 mM cycloheximide (Sigma) for 10 min at 30 °C.

Covalent labeling of SENPs with HA-SUMO-VME

In vitro synthesized ³⁵S-labeled full-length SENPs (0.5–1 μl), proteins comprising their catalytic domains alone (0.02–0.1 μl) or HeLa cell total homogenates (200 μg of total proteins, prepared as described [40]) were incubated with 0.2–0.4 μM HA-SUMO₂-VME for 15 min at 25 °C in 20 μl (final volume) of buffer A supplemented with 2 mM EDTA-NaOH, pH 8.0, 0.15 μg/μl BSA and 1 mM DTT. Reactions were stopped with Laemmli sample buffer and analyzed by SDS-PAGE/autoradiography.

RanGAP₁ monoSUMOylation assay

Full-length RanGAP₁ was synthesized in vitro for 45 min and treated with 1 mM cycloheximide for 10 min at 30 °C to prevent further protein synthesis. Samples were then diluted twofold with buffer A supplemented with 1 mM DTT, 2 mM MgCl₂ and 2 mM ATP (final concentrations) and incubated for 40 min at 37 °C in the presence of 50 nM E₁, 100 nM Ubc9 and 1 μM of either SUMO₁ or SUMO₂ or SUMO₃. SUMOylation reactions were terminated by depleting ATP with 5 U/ml (final concentration) of apyrase (grade VII, Sigma) for 10 min at 30 °C.

Densitometric analyses

In vitro synthesized full-length SENP₁, HeLa cell total proteins and different amounts of recombinant full-length SENP₁ were analyzed by SDS-PAGE/western-blot using an anti-SENP₁ antibody. The approximate levels of ³⁵S-labeled SENP₁ produced in the rabbit reticulocyte lysate (RRL) and of the endogenous enzyme in HeLa cells were determined by direct comparison with the recombinant protein, as assessed by densitometric analyses of western-blots using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997–2014). To estimate the relative stoichiometries of the in vitro synthesized SENPs to be used in the activity assays (Section 2.5.), aliquots of the RRLs containing the ³⁵S-labeled proteins were subjected to SDS-PAGE/autoradiography followed by densitometry analyses, taking into account the methionine content of each enzyme. Full-length SENP_{1/2/3/5/6/7} contain 14, 15, 15, 17, 14 and 14 methionines, respectively, whereas the corresponding catalytic domains contain 11, 9, 10, 7, 10 and 6 methionine residues, respectively.

SENP activity assays

The activity of in vitro synthesized SENPs was assessed by HA-SUMO₂-VME labeling. Approximate equimolar amounts of each SENP (see Section 2.4.) were then used in the activity assays unless otherwise indicated. The total volume of RRL used in each reaction was made equal by addition of a mock-translated and cycloheximide-treated RRL. A control RRL containing an in vitro synthesized unrelated protein, ³⁵S-labeled ΔC1-PEX5 [43], was also included in many assays as specified. The activity assays were performed at 25 °C in the presence of 0.6–2 μl of monoSUMOylated RanGAP₁ or 250 ng of polySUMO chains in 10 μl of buffer A supplemented with 2 mM EDTA-NaOH, pH 8.0, 0.15 μg/μl BSA and 2 mM DTT. For the activity assays with SUMO precursors, 200–500 ng of proSUMOs were directly added to 6 μl of normalized RRLs (i.e., mock plus SENP-containing RRL) and incubated at 25 °C. The approximate concentrations of SENP₁ in these assays were 6–7 nM, 3–5 nM and 1–2 nM for proSUMOs, RanGAP₁-SUMOs and polySUMO chains, respectively, as judged from the determinations with the anti-SENP₁ antibody (Section 2.4.). In the activity assays containing the catalytic domains and SENP7 mutants, the incubation times and/or amounts of

enzyme used were adjusted to obtain partial substrate hydrolysis. SENP-containing RRLs used in the assays with monoSUMOylated RanGAP1 were pretreated with apyrase (see Section 2.3.) to prevent reactivation of the exogenously added SUMO-conjugation machinery. Reactions from the activity assays with RanGAP1–SUMO were stopped with Laemmli sample buffer, separated in 8 or 12% SDS-PAGE and blotted onto a nitrocellulose membrane, followed by autoradiography to detect the radiolabeled proteins (SENPs, RanGAP1 and $\Delta C1$ -PEX5). In the activity assays with SUMO precursors and polySUMO chains, two aliquots of each sample were withdrawn after stopping the reactions. One aliquot was resolved in 16% SDS-PAGE and analyzed by western-blot with anti-SUMO1 or anti-SUMO2/3 antibodies to detect proSUMOs, polySUMO chains and their hydrolysis products. The second aliquot was analyzed by 8% SDS-PAGE/autoradiography to detect the radiolabeled SENPs. The stoichiometric ratios of SENPs were then reassessed by densitometry analyses (see Section 2.4.) and are presented in the figures. The contribution to SENP2 and SENP3 intensity signals from the band of the RanGAP1 doublet that co-migrates with those enzymes in some gels was estimated and subtracted by analyzing the intensity of the other RanGAP1 band and the stoichiometry of the RanGAP1 doublet in other lanes. All assays were performed at least three times; representative experiments are shown.

Antibodies

Mouse monoclonal anti-SUMO1 (21C7) [5] and anti-SUMO2/3 (8A2) [48] antibodies were developed by Michael J. Matunis and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242, USA. Rabbit polyclonal anti-SENP1 antibody (A302-927A-1) was purchased from Bethyl Laboratories, Inc. These antibodies were detected on western-blots using alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgGs (Sigma).

Results

As shown previously [40], full-length human SENPs synthesized in a RRL-based in vitro transcription/translation system are quite active as assessed by their reactivity toward the activity-based irreversible inhibitor SUMO2 vinyl methyl ester (SUMO2-VME; see Fig. 1A). Furthermore, the amounts of protein produced by the RRL, although orders of magnitude lower than those typically obtained using in vivo expression systems, are considerable. Indeed, as shown in Fig. 1B, 1 μ l of RRL produces approximately 3 ng of SENP1, an amount similar to that present in 100–200 μ g of total proteins from HeLa cells. These two findings led us to conclude that in vitro synthesized SENPs might be used in enzymatic assays aiming at better understanding their substrate specificities and relative activities. The strategy developed for this purpose turned out to be very simple because the endogenous SUMO protease activity of the RRL is relatively low. Indeed, with very few exceptions (e.g., see Fig. 2C, lower right panel), the activities detected in RRLs containing a negative control protein were always much lower than those detected in RRLs containing SENPs. This obviated the need to purify SENPs from the RRL before proceeding with the activity assays. Thus, aliquots of the in vitro translation reactions were simply mixed with a test substrate in an appropriate buffer and incubated (see Section 2.5. for details). Because one of our aims was also to gather qualitative information on the relative activities of the different SENPs, their relative stoichiometries in each of the experiments presented below were estimated by densitometric analyses of autoradiographs (see legends to figures and Section 2.4. for details). Fig. 2A shows the results obtained with SUMO1/2/3 precursors. From all human SENPs, only SENP1 and SENP2 show detectable processing

activity, with the former being more active on proSUMO₁ and the latter preferring proSUMO₂ and proSUMO₃. We next assessed the isopeptidase activity of the different SENPs using as a substrate monoSUMOylated RanGAP₁, a nuclear pore protein and a canonical target of SUMOylation [5,49]. Radiolabeled RanGAP₁ modified with SUMO₁ or SUMO₂ was produced in vitro (Fig. 2B; see Section 2.3.) and incubated with radiolabeled SENPs. Note that RanGAP₁ migrates in SDS-gels as a double band, a result of phosphorylation events occurring in the RRL [50]. As shown in Fig. 2C (upper left panel), SENP₁ is by far the most active isopeptidase achieving complete deconjugation of RanGAP₁–SUMO₁ in 5 min or less. SENP₂ also hydrolyzes the RanGAP₁–SUMO₁ conjugate but this activity was detectable only after a 1-h incubation (upper right panel). Different results were obtained with RanGAP₁–SUMO₂ (Fig. 2C, lower panels). In this case, SENP₁ is again the most active isopeptidase, but SENP₂ now shares with SENP₅ the second largest activity (left panel). No hydrolysis of RanGAP₁–SUMO₂ was detected in the 5 min incubation assay when testing SENP₃, SENP₆ and SENP₇ (Fig. 2C, left panel). A longer incubation did reveal some hydrolysis of this substrate in the SENP_{3/6/7}-containing RRLs. However, the amounts of RanGAP₁–SUMO₂ hydrolyzed in these samples are similar to or, at best, only slightly larger than the one obtained with a negative control RRL (right panel, compare lane C with lanes 3, 6 and 7). Substituting RanGAP₁–SUMO₃ for RanGAP₁–SUMO₂ in these assays yielded the same results (data not shown).

Lastly, we tested human SENPs on polySUMO₂ chain disassembly. As shown in Fig. 3, all SENPs display at least some activity with this substrate. Interestingly, SENP₁ and SENP₂ seem to be at least as active (if not more) as SENP₆ and SENP₇, the two enzymes typically associated with polySUMO disassembly. Also, the activity of SENP₆ on polySUMO₂ chains seems larger than that of SENP₇. However, due to the fact that in vitro synthesis of both SENP₆ and SENP₇ also results in the synthesis of significant amounts of truncated fragments (some of which may be catalytically active) this observation should be taken with caution. SENP₃ and SENP₅ are the least efficient SENPs in polySUMO₂ chain disassembly (lower panel, lanes 3 and 5, respectively). The same results were obtained with polySUMO₃ chains (data not shown). As stated above (Section 1.) there are some data suggesting that the non-catalytic N-terminal domains of SENPs may somehow modulate the activities of these enzymes. The availability of active full-length human SENPs prompted us to compare their activities with those of the corresponding catalytic domains. The catalytic domains of SENP_{1/2/5/6/7} used in these experiments were those defined before by other authors [30] (see Section 2.1.). For SENP₃, the previously defined catalytic domain (residues ³⁵³–574; [30]) turned out to be inactive, as assessed by reaction with SUMO₂-VME (data not shown), and thus we used instead a protein comprising residues 307–574 of SENP₃, the shortest SENP₃ protein tested that reacted quantitatively with SUMO₂-VME (see Fig. 4A). Note that the experimental conditions used in these assays for each SENP/catalytic domain pair were not the same and differ from those used in Fig. 3. Specifically, the incubation times and/or the amounts of enzyme in the assays were changed in order to avoid less informative situations of complete substrate hydrolysis. The results of this comparison using polySUMO₂ chains as substrate are shown in Fig. 4B. No obvious differences between the activities of full-length SENP₂ and SENP₃ and their catalytic domains were observed in these experiments. For SENP₁ we did observe a larger activity with its catalytic domain than with the full-length enzyme. This difference, however, is modest, as increasing the amount of full-length SENP₁ in the assay by a factor of ~2 yields approximately the activity observed for its catalytic domain. Furthermore, no difference in the activities of full-length SENP₁ and its catalytic domain was detected when using RanGAP₁–SUMO₂ as substrate (Fig. 4C). In contrast, marked differences between the activities of full-length enzymes and their catalytic domains were observed for SENP₅, SENP₆ and SENP₇ with polySUMO₂ chains,

but in these cases in the opposite direction (Fig. 4B). That is, their catalytic domains are much less active than the corresponding full-length enzymes. Again, for the reasons stated above these differences should be taken with caution in the cases of SENP6 and SENP7 (see also below). The lower activity of SENP5 catalytic domain compared to the full-length enzyme is also easily detectable when using RanGAP1–SUMO2 as a substrate (Fig. 4C). The non-catalytic N-terminal domains of several SENPs contain one or more SIMs [51]. These SIMs might act as substrate recruiting platforms and thus they could explain why full-length SENP5/6/7 are more active than the corresponding catalytic domains in the polySUMO2 hydrolysis assays presented above. To test this possibility, we used a SENP7 mutant protein that lacks seven of these SIMs and which was recently shown to be rather inefficient in homologous recombination DNA repair, a SUMO-dependent process that requires catalytically active SENP7 [41]. Unexpectedly, as shown in Fig. 5B, the mutant SENP7 protein is as efficient as SENP7 in disassembling polySUMO2 chains. Apparently, these SENP7 SIMs are not relevant for the enzyme activity, at least in these in vitro assays.

Discussion

From the qualitative assessment of SENP activities presented in this work (see Table 1) it is clear that SENP1 is one of the most active SENPs and the only one for which we could detect activity with all the substrates tested. Two observations are worth noting. The first is the almost exclusive capacity of SENP1 to cleave the two SUMO1-containing substrates used here. Indeed, under our experimental conditions, no other SENP cleaved proSUMO1 and only SENP2 displayed some activity on RanGAP1–SUMO1. This finding should not be taken as evidence to conclude that only SENP1 is capable of cleaving proSUMO1—as shown before, recombinant full-length SENP2 as well as the catalytic domains of SENP2 and SENP5 is also able to cleave proSUMO1 in vitro, when used at concentrations several fold larger than the ones used here [19,30,32,33,35]. What is apparent from our results is that SENP1 is by far the most efficient SENP acting on SUMO1-containing substrates, a finding re-enforcing the idea that this protease plays an essential role in all processes involving SUMO1 [29,52]. The second interesting result regards the activity of full-length SENP1 on polySUMO2/3 chain disassembly. Previous studies have shown that the catalytic domain of SENP1 is more active on polySUMO2 chain hydrolysis than those of SENP6 and SENP7 [22,53], but whether or not these findings were valid for the full-length enzymes remained unknown. Here, we show that full-length SENP1 is one of the most active enzymes, if not the most efficient, towards polySUMO2/3 chains. Furthermore, our data suggest that this is probably an intrinsic property of its catalytic domain (see Fig. 4B). Thus, the idea that disassembly of polySUMO2/3 chains is a function performed mainly by SENP6 and SENP7 [2,15,54], seems unlikely. SENP2, the enzyme more closely related to SENP1, also presents a large catalytic activity and broad substrate specificity but, in contrast to SENP1, displays a strong preference for SUMO2/3-containing substrates. These findings are consistent with data reported previously for the recombinant catalytic domain of this protease [30,35], as well as with the fact that Senp2 knockout mouse embryos present a strong accumulation of SUMO2/3 conjugates and depletion of free SUMO2/3, while the levels of free and conjugated SUMO1 remain essentially normal [27]. Our data also suggest that full-length SENP2, and not SENP1, is the most active SENP acting on proSUMO2 and proSUMO3, a result that differs from previous data showing that the catalytic domain of SENP1 is slightly more active than that of SENP2 in cleaving proSUMO2/3 [30]. Regardless of this difference, which given the non-quantitative nature of the assays used here and in that study may in fact be not significant, it is clear that SENP1 and SENP2 are by far the most active enzymes acting on proSUMO2/3. Due to

difficulties in obtaining stable and active recombinant versions of SENP₃, the catalytic properties of this SENP have remained poorly characterized. Indeed, besides some data showing that SENP₃ reacts with SUMO_{2/3}-VS but not with SUMO₁-VS [38–40] and prefers SUMO_{2/3}-modified substrates [20,39,55], very little was known. Here we show that although the in vitro synthesized full-length SENP₃ is fully active, as assessed by its quantitative reaction with HA-SUMO₂-VME, the enzyme displays only a minor isopeptidase activity. This finding is intriguing when we consider that knockdown of SENP₃ in human/mouse cells leads to a strong accumulation of SUMO_{2/3} conjugates [25,28], a phenomenon consistent with SENP₃ controlling the global levels of SUMO_{2/3}-ylated conjugates. One possibility to explain this paradox is to assume that our assay lacks some SENP₃-activating factor. If so, our data might suggest that this activating factor does not act by a de-repressing mechanism on its non-catalytic N-terminal domain because the catalytic domain of SENP₃ alone also displays a minor isopeptidase activity. Another possibility is to assume that SENP₃ acts on a large number of substrates but that all these substrates have some SENP₃-interacting domain which, clearly, does not exist in the substrates used here. Clearly, further data are necessary to understand the properties of SENP₃. SENP₅ is as efficient as SENP₂ in hydrolyzing RanGAP₁-SUMO_{2/3} but shows no detectable activity with RanGAP₁-SUMO₁ even after prolonged incubations, suggesting a very strong paralogue preference for SUMO_{2/3}. By contrast, it is one of the least active SENPs on polySUMO_{2/3} chains. This suggests that SENP₅ is probably more important in removing monoSUMO_{2/3} from SUMOylated substrates than in disassembling polySUMO_{2/3} chains. Interestingly, in contrast to the results on SENP_{1/2/3}, the catalytic domain of SENP₅ is considerably less active than the full-length enzyme. This suggests that the N-terminal non-catalytic domain of SENP₅ modulates its activity. Whether the N-terminal domain of SENP₅ increases its substrate affinity or the catalytic efficiency of the enzyme remains to be determined. As expected from previous findings [21,22,30,33], SENP₆ and SENP₇ display a clear preference for polySUMO_{2/3} chains with very little or no activity towards the other tested substrates. However, as stated above, our results also show that SENP₁ and SENP₂ are at least as active as SENP_{6/7}, suggesting that SENP_{6/7} are not alone in the task of disassembling polySUMO_{2/3} chains. As with SENP₅, we found that the catalytic domains of SENP₆ and SENP₇ seem to be less active than the corresponding full-length enzymes. Since all these SENPs possess putative SIMs at their non-catalytic N-terminal domains, we considered the possibility that their N-terminal domains might somehow contribute for substrate recruitment. Unexpectedly, we found that a biologically compromised SENP₇ mutant lacking seven of these SIMs [41] is as active as the normal protein in polySUMO₂ disassembly. Apparently, these SIMs are not important for SENP₇ polySUMO disassembly activity, at least in our assays. Naturally, given the difficulties in defining SIMs based solely on primary structure analyses (the SIM is quite a degenerate motif [51,56–58]), there may be additional SIMs in the N-terminal domain of SENP₇ playing some role in substrate recruitment. However, it is also possible that the larger activity of full-length SENP₇ in comparison with its catalytic domain is due to a stabilizing effect of its N-terminal domain over its catalytic domain, leading to an increase in the catalytic efficiency of the enzyme. Clearly, additional data are necessary to clarify these issues. In summary, here we describe a simple in vitro approach to study the properties of SENPs. The data collected, although of qualitative nature, provide a global perspective on the relative activities and substrates specificities of these biochemically intractable enzymes. Such an approach will be surely useful in any future work aiming at unraveling the structural/functional relationships of these proteases.

Acknowledgements

We thank Dr. Frauke Melchior (University of Heidelberg, Germany), Dr. Guy Salvesen (Sanford-Burnham Medical Research Institute, USA), Dr. Hidde Ploegh (Whitehead Institute, USA) and Dr. Joanna Morris (University of Birmingham, UK) for kindly providing plasmids. This work was funded by FEDER (Fundo Europeu de Desenvolvimento Regional) funds through the Operational Competitiveness Programme— COMPETE and by National Funds through FCT— Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-027627 (EXPL/BEX-BCM/0320/2012) and by project “NORTE-07-0124-FEDER000003- Cell homeotasis tissue organization and organism biology” co-funded by Programa Operacional Regional do Norte (ON.2—O Novo Norte), under the Quadro de Referência Estratégico Nacional (QREN), through FEDER and by FCT. A. V. M. was supported by project FCOMP01-0124-FEDER-027627-EXPL/BEX-BCM/0320/2012. C. P. G. (SFRH/ BPD/64388/2009) and M. P. P. (SFRH/BPD/47447/2008) were supported by FCT, COMPETE, Programa Operacional Potencial Humano (POPH) do QREN, FEDER and Fundo Social Europeu (FSE).

REFERENCES

- [1] A. Flotho, F. Melchior, Sumoylation: a regulatory protein modification in health and disease, *Annu. Rev. Biochem.* 82 (2013) 357–385, <http://dx.doi.org/10.1146/annurev-biochem-061909-093311>.
- [2] C.M. Hickey, N.R. Wilson, M. Hochstrasser, Function and regulation of SUMO proteases, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 755–766, <http://dx.doi.org/10.1038/nrm3478>.
- [3] A. Chen, H. Mannen, S.S. Li, Characterization of mouse ubiquitin-like SMT3A and SMT3B cDNAs and gene/pseudogenes, *Biochem. Mol. Biol. Int.* 46 (1998) 1161–1174.
- [4] T. Kamitani, K. Kito, H.P. Nguyen, T. Fukuda-Kamitani, E.T. Yeh, Characterization of a second member of the sentrin family of ubiquitin-like proteins, *J. Biol. Chem.* 273(1998) 11349–11353, <http://dx.doi.org/10.1074/jbc.273.18.11349>.
- [5] M.J. Matunis, E. Coutavas, G. Blobel, A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex, *J. Cell Biol.* 135 (1996) 1457–1470.
- [6] M.H. Tatham, E. Jaffray, O.A. Vaughan, J.M. Desterro, C.H. Botting, J.H. Naismith, R.T. Hay, Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9, *J. Biol. Chem.* 276 (2001) 35368–35374, <http://dx.doi.org/10.1074/jbc.M104214200>.
- [7] H. Saitoh, J. Hinchey, Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3, *J. Biol. Chem.* 275 (2000) 6252–6258, <http://dx.doi.org/10.1074/jbc.275.9.6252>.
- [8] J.M. Desterro, M.S. Rodriguez, G.D. Kemp, R.T. Hay, Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1, *J. Biol. Chem.* 274(1999) 10618–10624, <http://dx.doi.org/10.1074/jbc.274.15.10618>.



- [9] S.E. Schwarz, K. Matuschewski, D. Liakopoulos, M. Scheffner, S. Jentsch, The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 560–564.
- [10] E.S. Johnson, A.A. Gupta, An E3-like factor that promotes SUMO conjugation to the yeast septins, *Cell* 106 (2001) 735–744, [http://dx.doi.org/10.1016/S0092-8674\(01\)00491-3](http://dx.doi.org/10.1016/S0092-8674(01)00491-3).
- [11] J.R. Gareau, C.D. Lima, The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition, *Nat. Rev. Mol. Cell Biol.* 11 (2010) 861–871, <http://dx.doi.org/10.1038/nrm3011>.
- [12] I. Matic, M. van Hagen, J. Schimmel, B. Macek, S.C. Ogg, M.H. Tatham, R.T. Hay, A.I. Lamond, M. Mann, A.C. Vertegaal, In vivo identification of human small ubiquitin-like-modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy, *Mol. Cell. Proteomics* 7 (2008) 132–144, <http://dx.doi.org/10.1074/mcp.M700173-MCP200>.
- [13] M. Drag, G.S. Salvesen, DeSUMOylating enzymes—SENPs, *IUBMB Life* 60 (2008) 734–742, <http://dx.doi.org/10.1002/iub.113>.
- [14] R.T. Hay, SUMO-specific proteases: a twist in the tail, *Trends Cell Biol.* 17 (2007) 370–376, <http://dx.doi.org/10.1016/j.tcb.2007.08.002>.
- [15] D. Mukhopadhyay, M. Dasso, Modification in reverse: the SUMO proteases, *Trends Biochem. Sci.* 32 (2007) 286–295, <http://dx.doi.org/10.1016/j.tibs.2007.05.002>.
- [16] D. Bailey, P. O'Hare, Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1, *J. Biol. Chem.* 279 (2004) 692–703, <http://dx.doi.org/10.1074/jbc.M306195200>.
- [17] J. Goeres, P.K. Chan, D. Mukhopadhyay, H. Zhang, B. Raught, M.J. Matunis, The SUMO-specific isopeptidase SENP2 associates dynamically with nuclear pore complexes through interactions with karyopherins and the Nup107-160 nucleoporin subcomplex, *Mol. Biol. Cell* 22 (2011) 4868–4882, <http://dx.doi.org/10.1091/mbc.E10-12-0953>.
- [18] J. Hang, M. Dasso, Association of the human SUMO-1 protease SENP2 with the nuclear pore, *J. Biol. Chem.* 277 (2002) 19961–19966, <http://dx.doi.org/10.1074/jbc.M201799200>.
- [19] A. Di Bacco, J. Ouyang, H.Y. Lee, A. Catic, H. Ploegh, G. Gill, The SUMO-specific protease SENP5 is required for cell division, *Mol. Cell. Biol.* 26 (2006) 4489–4498, <http://dx.doi.org/10.1128/MCB.02301-05>.
- [20] L. Gong, E.T. Yeh, Characterization of a family of nucleolar SUMO-specific proteases with preference for SUMO-2 or SUMO-3, *J. Biol. Chem.* 281 (2006) 15869–15877, <http://dx.doi.org/10.1074/jbc.M511658200>.
- [21] D. Mukhopadhyay, F. Ayaydin, N. Kolli, S.H. Tan, T. Anan, A. Kametaka, Y. Azuma, K.D. Wilkinson, M. Dasso, SUSP1 antagonizes formation of highly SUMO2/3-conjugated species, *J. Cell Biol.* 174 (2006) 939–949, <http://dx.doi.org/10.1083/jcb.200510103>.
- [22] L.N. Shen, M.C. Geoffroy, E.G. Jaffray, R.T. Hay, Characterization of SENP7, a SUMO-2/3-specific isopeptidase, *Biochem. J.* 421 (2009) 223–230, <http://dx.doi.org/10.1042/BJ20090246>.

- [23] E.J. Shin, H.M. Shin, E. Nam, W.S. Kim, J.H. Kim, B.H. Oh, Y. Yun, DeSUMOylating isopeptidase: a second class of SUMO protease, *EMBO Rep.* 13 (2012) 339–346, <http://dx.doi.org/10.1038/embor.2012.3>.
- [24] S. Schulz, G. Chachami, L. Kozaczekiewicz, U. Winter, N. Stankovic-Valentin, P. Haas, K. Hofmann, H. Urlaub, H. Ovaa, J. Wittbrodt, E. Meulmeester, F. Melchior, Ubiquitin-specific protease-like 1 (USPL1) is a SUMO isopeptidase with essential, non-catalytic functions, *EMBO Rep.* 13 (2012) 930–938, <http://dx.doi.org/10.1038/embor.2012.125>.
- [25] C. Guo, K.L. Hildick, J. Luo, L. Dearden, K.A. Wilkinson, J.M. Henley, SENP3-mediated deSUMOylation of dynamin-related protein 1 promotes cell death following ischaemia, *EMBO J.* 32 (2013) 1514–1528, <http://dx.doi.org/10.1038/emboj.2013.65>.
- [26] N. Hattersley, L. Shen, E.G. Jaffray, R.T. Hay, The SUMO protease SENP6 is a direct regulator of PML nuclear bodies, *Mol. Biol. Cell* 22 (2011) 78–90, <http://dx.doi.org/10.1091/mbc.E10-06-0504>.
- [27] X. Kang, Y. Qi, Y. Zuo, Q. Wang, Y. Zou, R.J. Schwartz, J. Cheng, E.T. Yeh, SUMO-specific protease 2 is essential for suppression of polycomb group protein-mediated gene silencing during embryonic development, *Mol. Cell* 38 (2010) 191–201, <http://dx.doi.org/10.1016/j.molcel.2010.03.005>.
- [28] M.H. Lee, A.M. Mabb, G.B. Gill, E.T. Yeh, S. Miyamoto, NF- κ B induction of the SUMO protease SENP2: a negative feedback loop to attenuate cell survival response to genotoxic stress, *Mol. Cell* 43 (2011) 180–191, <http://dx.doi.org/10.1016/j.molcel.2011.06.017>.
- [29] P. Sharma, S. Yamada, M. Lualdi, M. Dasso, M.R. Kuehn, Senp1 is essential for desumoylating Sumo1-modified proteins but dispensable for Sumo2 and Sumo3 deconjugation in the mouse embryo, *Cell Rep.* 3 (2013) 1640–1650, <http://dx.doi.org/10.1016/j.celrep.2013.04.016>.
- [30] J. Mikolajczyk, M. Drag, M. Bekes, J.T. Cao, Z. Ronai, G.S. Salvesen, Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs, *J. Biol. Chem.* 282 (2007) 26217–26224, <http://dx.doi.org/10.1074/jbc.M702444200>.
- [31] Z. Xu, S.W. Au, Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1, *Biochem. J.* 386 (2005) 325–330, <http://dx.doi.org/10.1042/BJ20041210>.
- [32] H. Zhang, H. Saitoh, M.J. Matunis, Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex, *Mol. Cell Biol.* 22 (2002) 6498–6508, <http://dx.doi.org/10.1128/MCB.22.18.6498-6508.2002>.
- [33] C.D. Lima, D. Reverter, Structure of the human SENP7 catalytic domain and polySUMO deconjugation activities for SENP6 and SENP7, *J. Biol. Chem.* 283 (2008) 32045–32055, <http://dx.doi.org/10.1074/jbc.M805655200>.
- [34] D. Reverter, C.D. Lima, A basis for SUMO protease specificity provided by analysis of human Senp2 and a Senp2–SUMO complex, *Structure* 12 (2004) 1519–1531, <http://dx.doi.org/10.1016/j.str.2004.05.023>.
- [35] D. Reverter, C.D. Lima, Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates, *Nat. Struct. Mol. Biol.* 13 (2006) 1060–1068, <http://dx.doi.org/10.1038/nsmb1168>.

- [36] L. Shen, M.H. Tatham, C. Dong, A. Zagorska, J.H. Naismith, R.T. Hay, SUMO protease SENP1 induces isomerization of the scissile peptide bond, *Nat. Struct. Mol. Biol.* 13 (2006) 1069–1077, <http://dx.doi.org/10.1038/nsmb1172>.
- [37] L.N. Shen, C. Dong, H. Liu, J.H. Naismith, R.T. Hay, The structure of SENP1–SUMO-2 complex suggests a structural basis for discrimination between SUMO paralogues during processing, *Biochem. J.* 397 (2006) 279–288, <http://dx.doi.org/10.1042/BJ20052030>.
- [38] N. Kolli, J. Mikolajczyk, M. Drag, D. Mukhopadhyay, N. Moffatt, M. Dasso, G. Salvesen, K.D. Wilkinson, Distribution and paralogue specificity of mammalian deSUMOylating enzymes, *Biochem. J.* 430 (2010) 335–344, <http://dx.doi.org/10.1042/BJ20100504>.
- [39] C. Yun, Y. Wang, D. Mukhopadhyay, P. Backlund, N. Kolli, A. Yergey, K.D. Wilkinson, M. Dasso, Nucleolar protein B23/nucleophosmin regulates the vertebrate SUMO pathway through SENP3 and SENP5 proteases, *J. Cell Biol.* 183 (2008) 589–595, <http://dx.doi.org/10.1083/jcb.200807185>.
- [40] M.P. Pinto, A.F. Carvalho, C.P. Grou, J.E. Rodriguez-Borges, C. Sa-Miranda, J.E. Azevedo, Heat shock induces a massive but differential inactivation of SUMO-specific proteases, *Biochim. Biophys. Acta* 1823 (2012) 1958–1966, <http://dx.doi.org/10.1016/j.bbamcr.2012.07.010>.
- [41] A.J. Garvin, R.M. Densham, S.A. Blair-Reid, K.M. Pratt, H.R. Stone, D. Weekes, K.J. Lawrence, J.R. Morris, The deSUMOylase SENP7 promotes chromatin relaxation for homologous recombination DNA repair, *EMBO Rep.* 14 (2013) 975–983, <http://dx.doi.org/10.1038/embor.2013.141>.
- [42] J. Joseph, S.H. Tan, T.S. Karpova, J.G. McNally, M. Dasso, SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles, *J. Cell Biol.* 156 (2002) 595–602, <http://dx.doi.org/10.1083/jcb.200110109>.
- [43] C.P. Grou, A.F. Carvalho, M.P. Pinto, S. Wiese, H. Piechura, H.E. Meyer, B. Warscheid, C. Sa-Miranda, J.E. Azevedo, Members of the E2D (UbcH5) family mediate the ubiquitination of the conserved cysteine of Pex5p, the peroxisomal import receptor, *J. Biol. Chem.* 283 (2008) 14190–14197, <http://dx.doi.org/10.1074/jbc.M800402200>.
- [44] A. Borodovsky, H. Ovaa, N. Kolli, T. Gan-Erdene, K.D. Wilkinson, H.L. Ploegh, B.M. Kessler, Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family, *Chem. Biol.* 9 (2002) 1149–1159, [http://dx.doi.org/10.1016/S1074-5521\(02\)00248-X](http://dx.doi.org/10.1016/S1074-5521(02)00248-X).
- [45] E. Meulmeester, M. Kunze, H.H. Hsiao, H. Urlaub, F. Melchior, Mechanism and consequences for paralogue-specific sumoylation of ubiquitin-specific protease 25, *Mol. Cell* 30 (2008) 610–619, <http://dx.doi.org/10.1016/j.molcel.2008.03.021>.
- [46] A. Pichler, A. Gast, J.S. Seeler, A. Dejean, F. Melchior, The nucleoporin RanBP2 has SUMO1 E3 ligase activity, *Cell* 108 (2002) 109–120, [http://dx.doi.org/10.1016/S0092-8674\(01\)00633-X](http://dx.doi.org/10.1016/S0092-8674(01)00633-X).
- [47] A. Werner, M.C. Moutty, U. Moller, F. Melchior, Performing in vitro sumoylation reactions using recombinant enzymes, *Methods Mol. Biol.* 497 (2009) 187–199, http://dx.doi.org/10.1007/978-1-59745-566-4_12.



- [48] X.D. Zhang, J. Goeres, H. Zhang, T.J. Yen, A.C. Porter, M.J. Matunis, SUMO-2/3 modification and binding regulate the association of CENP-E with kinetochores and progression through mitosis, *Mol. Cell* 29 (2008) 729–741, <http://dx.doi.org/10.1016/j.molcel.2008.01.013>.
- [49] R. Mahajan, C. Delphin, T. Guan, L. Gerace, F. Melchior, A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2, *Cell* 88 (1997) 97–107, [http://dx.doi.org/10.1016/S0092-8674\(00\)81862-0](http://dx.doi.org/10.1016/S0092-8674(00)81862-0).
- [50] E. Takeda, M. Hieda, J. Katahira, Y. Yoneda, Phosphorylation of RanGAP1 stabilizes its interaction with Ran and RanBP1, *Cell Struct. Funct.* 30 (2005) 69–80, <http://dx.doi.org/10.1247/csf.30.69>.
- [51] O. Kerscher, SUMO junction—what's your function? New insights through SUMOinteracting motifs, *EMBO Rep.* 8 (2007) 550–555, <http://dx.doi.org/10.1038/sj.embor.7400980>.
- [52] T. Yamaguchi, P. Sharma, M. Athanasiou, A. Kumar, S. Yamada, M.R. Kuehn, Mutation of SENP1/SuPr-2 reveals an essential role for desumoylation in mouse development, 146 A.V. Mendes et al. / *Biochimica et Biophysica Acta* 1863 (2016) 139–147 *Mol. Cell. Biol.* 25 (2005) 5171–5182, <http://dx.doi.org/10.1128/MCB.25.12.5171-5182.2005>.
- [53] M. Bekes, J. Prudden, T. Srikumar, B. Raught, M.N. Boddy, G.S. Salvesen, The dynamics and mechanism of SUMO chain deconjugation by SUMO-specific proteases, *J. Biol. Chem.* 286 (2011) 10238–10247, <http://dx.doi.org/10.1074/jbc.M110.205153>.
- [54] A.C. Vertegaal, SUMO chains: polymeric signals, *Biochem. Soc. Trans.* 38 (2010) 46–49, <http://dx.doi.org/10.1042/BST0380046>.
- [55] T. Nishida, H. Tanaka, H. Yasuda, A novel mammalian Smt3-specific isopeptidase 1 (SMT3IP1) localized in the nucleolus at interphase, *Eur. J. Biochem.* 267 (2000) 6423–6427, <http://dx.doi.org/10.1046/j.1432-1327.2000.01729.x>.
- [56] J.T. Hannich, A. Lewis, M.B. Kroetz, S.J. Li, H. Heide, A. Emili, M. Hochstrasser, Defining the SUMO-modified proteome by multiple approaches in *Saccharomyces cerevisiae*, *J. Biol. Chem.* 280 (2005) 4102–4110, <http://dx.doi.org/10.1074/jbc.M413209200>.
- [57] C.M. Hecker, M. Rabiller, K. Haglund, P. Bayer, I. Dikic, Specification of SUMO1- and SUMO2-interacting motifs, *J. Biol. Chem.* 281 (2006) 16117–16127, <http://dx.doi.org/10.1074/jbc.M512757200>.
- [58] J. Song, L.K. Durrin, T.A. Wilkinson, T.G. Krontiris, Y. Chen, Identification of a SUMObinding motif that recognizes SUMO-modified proteins, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 14373–14378, <http://dx.doi.org/10.1073/pnas.0403498101>.

Table 1. Summary of the relative activities and substrate specificities of SENPs.

The relative activities of the six SENPs for a given substrate are qualitatively classified from the maximum activity detected (+++) to non-detectable (-). Note that this classification cannot be used to compare the activity of a given SENP on different substrates because different experimental conditions were used for each substrate.

	SENP1	SENP2	SENP3	SENP5	SENP6	SENP7
ProS1	+++	-	-	-	-	-
ProS2	++	+++	-	-	-	-
ProS3	++	+++	-	-	-	-
RanGAP1-S1	+++	+	-	-	-	-
RanGAP1-S2/3	+++	++	-/+	++	-/+	-/+
PolyS2/3	+++	+++	+	+	+++	++

S1/2/3, SUMO1/2/3.

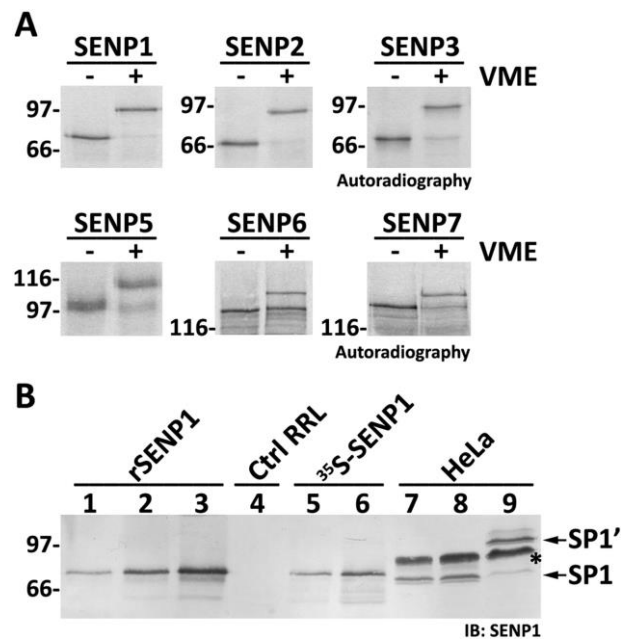


Fig. 1. In vitro synthesized full-length SENPs are active. A. ³⁵S-labeled full-length SENPs were synthesized in vitro and incubated at 25 °C in the absence (-) or presence (+) of HA-SUMO₂-VME (VME). Reactions were analyzed by SDS-PAGE/autoradiography. B. SENP₁ levels in HeLa cells and in the rabbit reticulocyte lysate. Increasing amounts of bacterially expressed recombinant full-length SENP₁ (rSEN1, lanes 1–3; 1.5, 3 and 6 ng, respectively), in vitro synthesized SENP₁ (³⁵S-SEN1, lanes 5–6; 0.5 and 1 μl, respectively) and HeLa cell total proteins (HeLa, lanes 7–8; 100 and 200 μg, respectively) were subjected to SDS-PAGE/western-blot and probed with an anti-SENP₁ antibody. The RRL synthesizes 3.2 ± 0.3 ng/μl of ³⁵S-labeled SENP₁ and HeLa cells contain 2.1 ± 0.6 ng of endogenous SENP₁ per 100 μg of total proteins, as determined by densitometric analysis (see Section 2.4.). An RRL containing an in vitro synthesized control protein (see Section 2.5.) was also analyzed (Ctrl RRL, lane 4; 1 μl), as well as an aliquot of HeLa cell total proteins (200 μg) previously subjected to HA-SUMO₂-VME labeling (lane 9). Unmodified (SP₁) and modified (SP₁') SENP₁ species are indicated. Asterisk, non-specific band detected by the anti-SENP₁ antibody in HeLa cells. Molecular masses of protein standards in kDa are shown to the left of the panels.

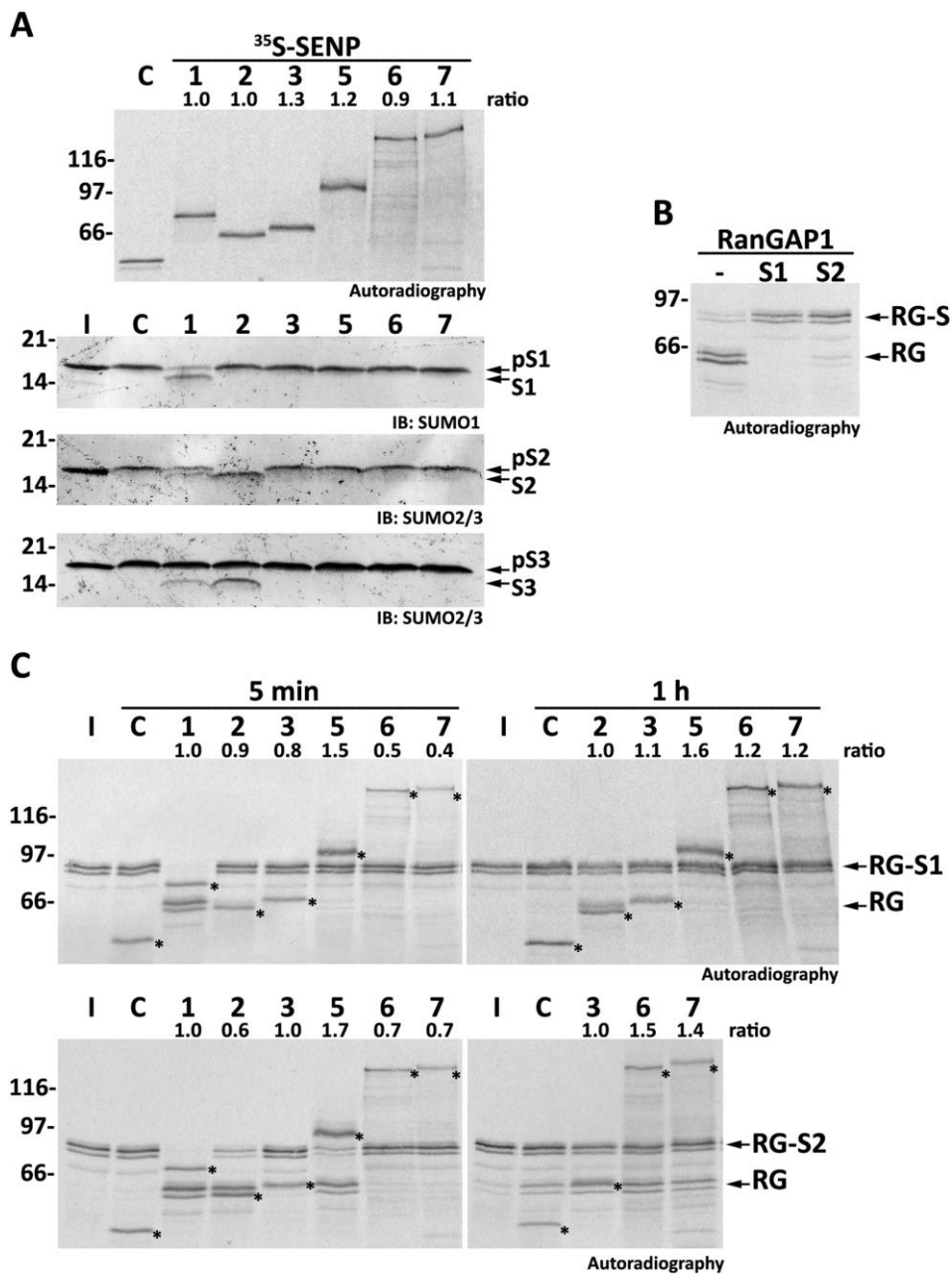


Fig. 2. Activity profiling of SENPs with SUMO precursors and monoSUMOylated substrates. A. Similar amounts of ³⁵S-labeled full-length SENP_{1-3/5-7} were incubated for 2 h at 25 °C with proSUMO₁ (pS₁), proSUMO₂ (pS₂) or proSUMO₃ (pS₃). At the end of the incubation, two aliquots of each sample were withdrawn. One aliquot was run on a 16% SDS-PAGE followed by western-blot with anti-SUMO₁ or anti-SUMO_{2/3} antibodies to detect both the SUMO precursors and their hydrolysis products SUMO₁ (S₁), SUMO₂ (S₂) and SUMO₃ (S₃). The second aliquot was subjected to 8% SDS-PAGE/autoradiography to detect the radiolabeled SENPs (1–7, upper panel). Their stoichiometric ratios as determined by densitometry analysis are also shown (see Section 2.4. for details). B. In vitro synthesized full-length RanGAP₁ (-) was subjected to SUMOylation with either

SUMO1 (S₁) or SUMO2 (S₂). Unconjugated (RG) and SUMOylated RanGAP1 (RG-S) are indicated. C. ³⁵S-labeled full-length SENPs (1–7) were incubated at 25 °C for 5 min (left panels) or 1 h (right panels) in the presence of RanGAP1–SUMO1 (RG–S₁, upper panels) or RanGAP1–SUMO2 (RG–S₂, lower panels). Samples were analyzed by SDS-PAGE/autoradiography to detect both RanGAP1 (RG) and SENPs (asterisks). Molar ratios of SENPs were determined as in B. Lanes C contain an irrelevant *in vitro* synthesized protein (asterisk) to control the activity of endogenous SENPs from the RRL. Lane I, SENP substrate used in each reaction.

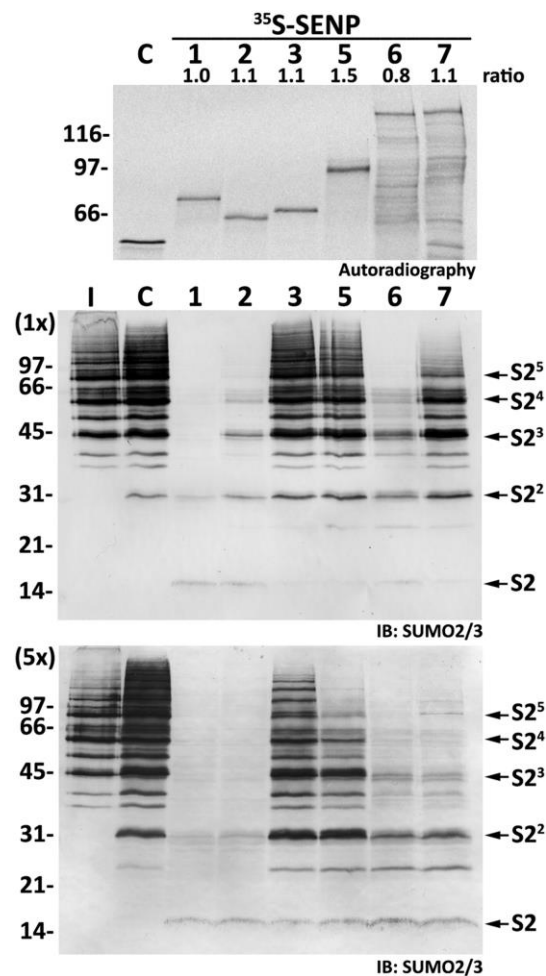


Fig. 3. PolySUMO₂ chain disassembly activity of SENPs. *In vitro* synthesized full-length SENPs (1–7, upper panel) were incubated for 2 h at 25 °C with polySUMO₂ chains (1 ×, middle panel). Similar reactions containing fivefold more SENPs (5 ×) were also prepared (lower panel). After stopping the reactions, two aliquots were withdrawn. One aliquot was analyzed in 16% SDS-PAGE followed by western-blot with an anti-SUMO_{2/3} antibody. SUMO₂ (S₂) and chains containing two to five SUMO moieties (S₂²–S₂⁵) are indicated. Note that monomeric SUMO₂ runs in these gels together with the very abundant hemoglobin protein from the reticulocyte lysate, partially hampering its detection in western-blot. The other aliquot was analyzed by 8% SDS-PAGE/autoradiography to detect radiolabeled SENPs (upper panel). Their stoichiometric ratios are also indicated (see legend to Fig. 2). An unrelated *in vitro* synthesized protein (C) was included in these assays to control for background activity. Lane I, polySUMO₂ chains used in each reaction.

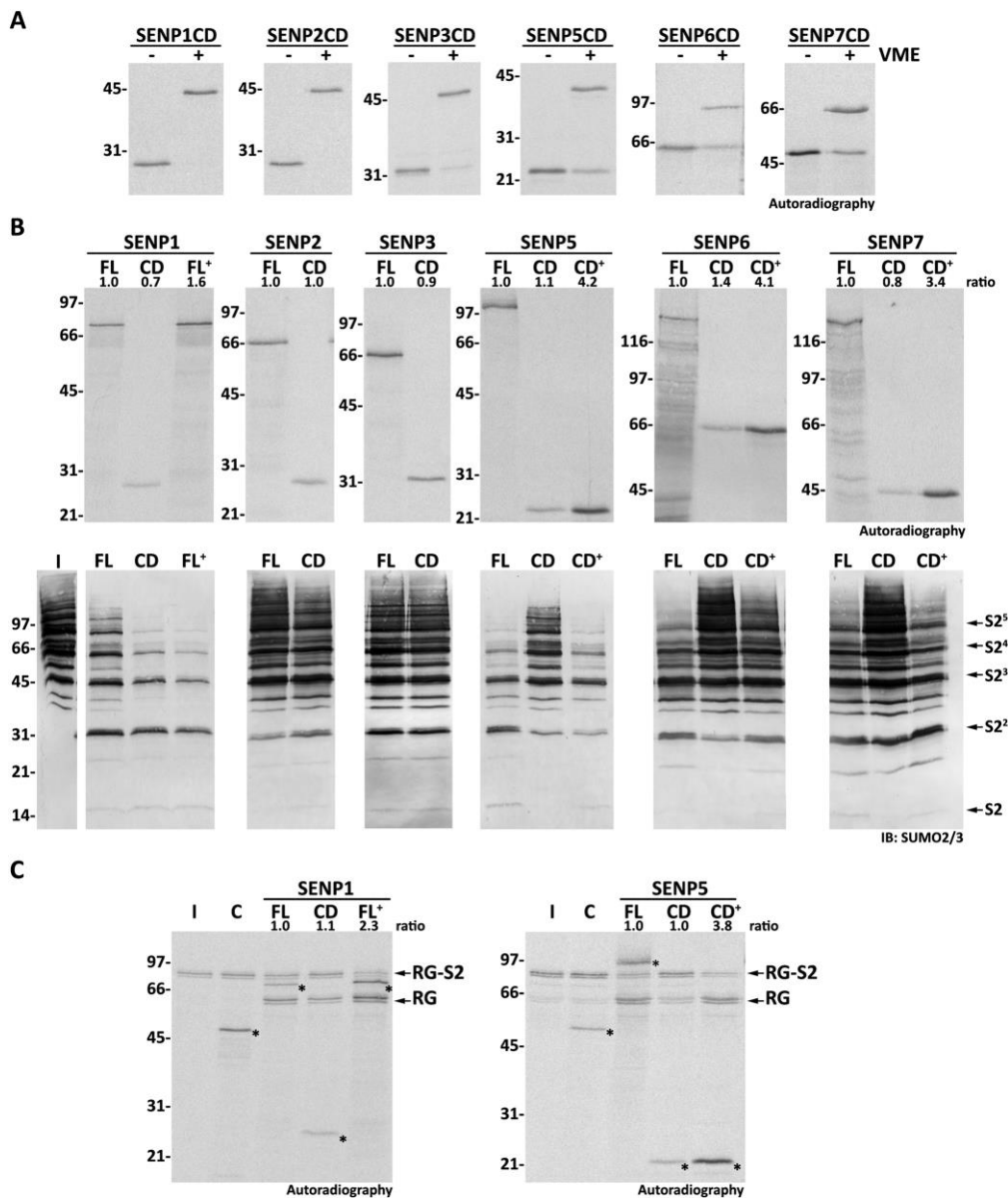


Fig. 4. Influence of the N-terminal domains of SENPs on their catalytic activity. A. ³⁵S-labeled versions of the catalytic domains (CD) of SENPs were synthesized in vitro and incubated in the absence (-) or presence (+) of HA-SUMO₂-VME (VME) followed by SDS-PAGE/autoradiography analysis. B. Full-length SENPs (FL) and similar stoichiometric amounts of their catalytic domains alone (CD) were incubated with polySUMO₂ chains for 5 min (SEN1/2) or 1 h (SEN3/5-7) at 25 °C. Some reactions contained a molar excess of full-length enzyme (FL+) or catalytic domain (CD+). Two aliquots were withdrawn from each sample. One aliquot was analyzed by 16% SDS-PAGE/western-blot with an anti-SUMO_{2/3} antibody to detect polySUMO₂ chains and their hydrolysis products (lower panels). SUMO₂ (S₂) as well as chains containing two to five SUMO₂ units (S₂₂-S₂₅) are indicated. The other aliquot was subjected to SDS-PAGE (8 or 12%)/autoradiography to detect the radiolabeled SENPs (upper panels). For each SENP, the stoichiometric ratios between the full-length enzyme and the catalytic domain are shown (see also

legend to Fig. 2). C. In vitro synthesized full-length SENP1/5 and their respective catalytic domains were incubated for 5 min at 25 °C using RanGAP1–SUMO₂ (RG–S₂) as substrate and analyzed by SDS-PAGE/autoradiography. SENP molar ratios are also shown. The radiolabeled enzymes as well as a control protein (C) are indicated by asterisks. Lane I, SENP substrate used in each lane.

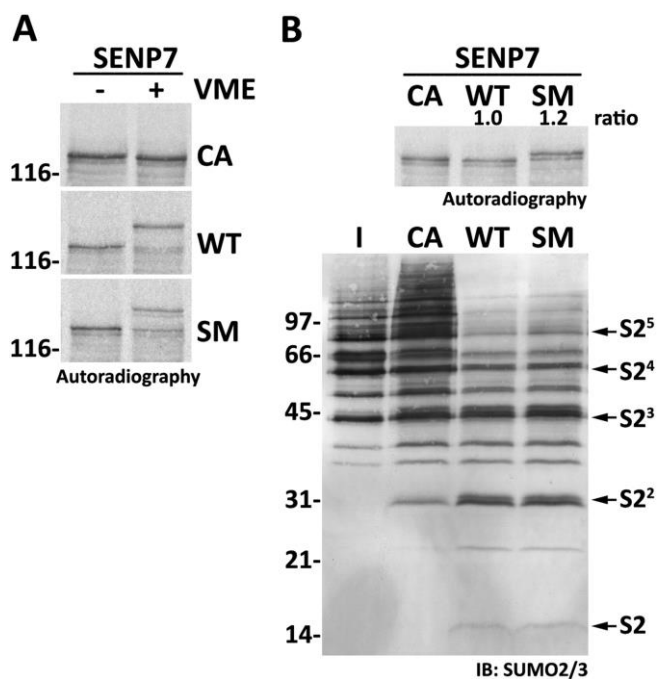


Fig. 5. A SENP7 mutant lacking seven putative SIMs is as active as the wild type enzyme on polySUMO₂ chain disassembly. A. Radiolabeled wild type SENP7 (WT), a SIM mutant (SM) and a catalytic inactive C992A mutant (CA) were synthesized in vitro, incubated at 25 °C in the absence (-) or presence (+) of HA-SUMO₂-VME (VME) and analyzed by SDS-PAGE/autoradiography. B. The wild type (WT), the SIM mutant (SM) and the catalytic inactive (CA) full-length versions of SENP7 were used in activity assays with polySUMO₂ chains for 1 h at 25 °C. An aliquot of each reaction was analyzed by 16% SDS-PAGE/western-blot with an anti-SUMO_{2/3} antibody (lower panel). SUMO₂ (S₂) and chains containing two to five SUMO moieties (S₂₂–S₂₅) are indicated. A second aliquot was subjected to 8% SDS-PAGE/autoradiography to detect the radiolabeled SENP7 versions used in the assays (upper panel). The stoichiometric ratio between wild type SENP7 and the SIM mutant is shown (see also legend to Fig. 2). Lane I, polySUMO₂ chains used in each reaction.