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Distribution and Paralog Specificity of Mammalian DeSUMOylating Enzymes

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SYNOPSIS

The covalent attachment of the small ubiquitin-like protein modifier (SUMO) to target proteins results in modifications in their activity, binding interactions, localization or half-life. The reversal of this modification is catalyzed by SUMO-specific processing proteases (SENPs). Mammals contain four SUMO paralogs and six SENP enzymes. Our studies describe a systematic analysis of human SENPs, integrating estimates of relative selectivity for SUMO1 and SUMO2, and kinetic measurements of recombinant C-terminal SENP catalytic domains (cSENPs). We first characterized the reaction of each endogenous SENP and their catalytic domains (cSENPs) with HA-tagged SUMO1 and SUMO2 vinyl sulfones (HA-SUMO-VS), active site-directed irreversible inhibitors of SENPs. We found that all cSENPs and endogenous SENP1 react with both SUMO paralogs, while all other endogenous SENPs in mammalian cells and tissues display high selectivity for SUMO2-VS. To obtain more quantitative data, the kinetic properties of purified cSENPs were determined using SUMO1 or SUMO2-amidomethyl coumarin (SUMO-AMC) as substrate. All enzymes bind their respective substrates with high affinity. cSENPs1 and cSENPs2 process either SUMO substrate with similar affinity and catalytic efficiency; cSENPs5 and cSENPs6 show marked catalytic specificity for SUMO2 as measured by K_M and k_{cat} while cSENPs7 works only on SUMO2. Compared to cSENPs, recombinant full-length SENP1 and SENP2 show differences in SUMO selectivity indicating that paralog specificity is influenced by the presence of the variable N-terminal domain of each SENP. Our data suggests that SUMO2 metabolism is more dynamic than that of SUMO1 since most SENPs display a marked preference for SUMO2.

Keywords

SUMO; paralogs; substrate specificity; SENP; desumoylation

INTRODUCTION

SUMO family proteins (Small Ubiquitin-like Protein Modifier) share a conserved ubiquitin like domain and a C-terminal di-glycine motif. The covalent modification of proteins by SUMO plays a dynamic role in transcriptional regulation, formation of PML (Pro-Myelocytic Leukaemia) nuclear bodies, nuclear transport, chromosome organization, DNA repair, mitotic progression and ribosome biogenesis [1–4].

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While *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* have a single SUMO, humans have four SUMO paralogs. Human SUMO2 and SUMO3 are well conserved and share 95% amino acid sequence identity. A key property of SUMO2 and SUMO3 is their ability to polymerize and form poly-SUMO chains [5]. SUMO4, another isoform, has been found to be 86% identical to SUMO2 and SUMO3 [6]. However, it has a very restricted tissue distribution and has not yet been shown to modify target proteins *in vivo*. SUMO1 is the most divergent family member, sharing 46% identity with SUMO2 and SUMO3.

Recent proteomic studies have identified many proteins modified by SUMO [7–11]. The best-studied are RanGap1 and topoisomerase II, predominantly modified by SUMO1 and SUMO2/3 respectively [12, 13]. Many SUMO-conjugated proteins are found in the nucleus, suggesting that SUMOylation is an important nuclear process [14]. In fact, SUMO2 and SUMO3 locate to the nucleoplasm while SUMO1 is distributed to the nuclear envelope and the nucleolus [15].

The covalent addition of SUMO to a target protein (SUMOylation) forms an isopeptide bond between the C-terminus of mature SUMO and the ϵ -amino group of the target lysine in the substrate protein. SUMOylation is catalyzed by a specific SUMO-E1 activating enzyme, the heterodimeric SAE1/SAE2, one SUMO E2 conjugating enzyme, Ubc9, and various SUMO-E3 ligases (Pc2, Siz1, RanBP2/Nup358, etc.). This is a reversible modification regulated by both SUMOylating and deSUMOylating enzymes (SENPs). SENPs also have an endopeptidase (C-terminal hydrolase) activity that is essential to process SUMO precursor proteins to expose the C-terminal di-glycine motif and yield mature SUMO.

Six SUMO-specific SENPs have been identified in humans, SENP1, 2, 3, 5, 6 and 7. Most SENPs possess both isopeptidase and endopeptidase activity, and it is not yet clear which activity is most essential for each SENP [16]. Evolutionary relationships divide SENPs into two major groups: the Ulp1p group (consisting of two sub-families, SENP1–2, and SENP3–5) and the Ulp2p group (SENP6–7) [17]. These genetic distinctions are also reflected in the specificity of the enzymes ([18, 19], this study); SENP1 and 2 catalyze hydrolysis of both SUMO1 and 2/3 derivatives [20, 21], SENP3 and 5 prefer SUMO2/3 [22] and SENP6 and 7 are thought to work best on polySUMO2/3 [23, 24]. A seventh Ulp family protease, SENP8 (also known as Den1 or NEDP1), shares marked similarity in the SENP catalytic domain, but lacks a SUMO-binding site and instead shows specificity for NEDD8 [25–28].

SENPs share a conserved C-terminal cysteine protease domain of approximately 250 amino acids containing the catalytic triad His/Asp/Cys (Figure 1). The SENP N-terminal domain is not well conserved and although there are essentially no data on this domain, it is believed to be responsible for substrate specificity and/or localization [29]. The SENP N-terminal domains vary in primary sequence and size [30]. SENP1 and SENP2 have nuclear localization signals (NLS) and nuclear export signals (NES) that likely play a role in the regulation of cellular localization, along with other potential regulatory motifs which vary for each individual SENP [29]. SENP1 localizes to the nuclear foci and the nuclear rim [31]. SENP2 exists in three splice variants that localize differently: SENP2/axam to the nuclear side of the nuclear pore complex [32, 33] Smt3IP2/Axam2 to the cytoplasm [34]; and SuPr-1 to PML oncogenic domains [35]. Both SENP3 and SENP5 localize to the nucleolus [22, 36], while SENP6 and SENP7 are both found in the nucleoplasm [23, 24, 37].

Previous studies indicate that SENPs might be highly target-specific and non-redundant *in vivo*. SENP1, SENP2 and SENP3 function by modulating nucleo-cytoplasmic shuttling [38–40]. SENP1 and SENP2/SuPr-1 regulate c-jun dependent transcription [35, 41]. SENP5 plays a physiological role in cell division and cell proliferation [42]. Both SENP3 and

SEN5 interact with B23/nucleophosmin *in vivo* to promote ribosome synthesis [4, 43] whereas SEN6 and 7 appear to regulate PML metabolism *in vivo* [23, 24]. Thus, substrate specificity is a consequence of SENP expression levels, the localization or co-localization with substrates and their inherent capability to distinguish different SUMO paralogs. Nonetheless the functional role of different SENPs in cellular processes is still poorly understood.

In the present study we have concentrated on characterizing the paralog selectivity of mammalian SENPs. HA-tagged SUMO-vinylsulfones (HA-SUMO-VS) were used as active-site directed irreversible inhibitors to identify binding and catalytic preference of SENPs for SUMO1 or SUMO2. Additionally, amidomethylcoumarin derivatives of SUMO (SUMO-AMC) are used as substrates to determine SENP isopeptidase activity and catalytic specificity. We examined both full length endogenous SENPs in lysates to identify isoform selectivity and recombinant C-terminal catalytic domains to measure and compare catalytic constants. While all recombinant cSENPs react with either SUMO-VS paralog, endogenous SENP2, 3, 5, 6 and 7 show distinct preferential selectivity for SUMO2 over SUMO1. We also observed that the non-conserved N-terminal domain of SENP2 plays a significant role in SENP selectivity.

EXPERIMENTAL

Preparation of vinyl sulfone (VS) and amido-4-methylcoumarin (AMC) reagents

HA-SUMO1-VS, HA-SUMO2-VS, SUMO1-AMC and SUMO2-AMC were prepared using intein fusion protein technology as detailed previously [44]. SUMO1 and SUMO2 expression, purification, and concentration was monitored by SDS PAGE analysis and high performance liquid chromatography (HPLC) on a C8 reverse phase column (buffers A: 25mM NaClO₄, 0.07% HClO₄ and B: buffer A plus 75 % acetonitrile with a 0–80% gradient over 25 minutes monitoring at 205nm). MALDI MS analysis confirmed the purity and identity of the vinyl, sulfone and AMC derivatives. All masses were consistent with the expected mass within $\pm 0.1\%$.

Protein expression and purification

Full-length pGEX-Ulp1 and pGEX-Ulp1_{403–621} were expressed in BL21 cells induced with 0.5mM isopropyl-beta-D-thiogalactopyranoside (IPTG) during a 3-hour induction at room temperature. Expressed pGEX-Ulp1 protein was isolated with glutathione-agarose resin (Sigma) and pGEX-Ulp1_{403–621} on Ni-NTA agarose (Invitrogen) respectively. The recombinant catalytic domains of SENPs 1, 2, 3, 5, 6, and 7 and His₆-tagged full-length SENP1 and SENP2 were expressed in E. coli and purified as described previously [16]. Purification of all proteins was evaluated by SDS PAGE.

Mammalian cell lysates and rabbit tissue extracts

HEK293, HeLa, LM TK⁻, A9, and Cos-7 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO₂ incubator. Cells were lysed by sonication in lysis buffer (50mM Tris-HCl pH 7.4, 5mM MgCl₂, 250mM sucrose, 1mM dithiothreitol, 2mM ATP) with protease inhibitors (Roche diagnostics, IN, USA). The cell lysates were clarified by centrifugation at 20,000 × g for 30 minutes. Rabbit thymus, spleen, brain, heart, adrenal and thyroid gland (Pel freeze) were homogenized in lysis buffer with protease inhibitors. Extracts were clarified initially by centrifugation at 20,000 × g for 15 minutes followed by 36,000 × g for 1 hour.

Steady-state kinetic analysis

Enzyme activity assays for cSENPs and cell lysates were performed as previously described [45] using a SUMO C-terminal 7-amido-4-methylcoumarin substrate (SUMO-AMC) [44]. In a typical assay, 140 μ l of assay buffer (50mM Tris-HCl, pH 7.8, 100 μ g/ml ovalbumin, 10mM DTT) containing variable concentrations of SUMO1-AMC or SUMO2-AMC and purified cSENPs were added to the reaction mix. The reaction was monitored by measuring the increase in fluorescence at 440 nm ($\lambda_{\text{ex}} = 340$ nm) using an Aminco Bowman Series 2 fluorimeter. Activity assays were conducted at 37°C in triplicate. The SUMO-AMC preparations contain approximately 10% unlabeled SUMO (data not shown). The simplest model of Michaelis and Menten was used to fit the rate of enzyme activity as a function of substrate concentration. The initial rates obtained with each enzyme were compared and the apparent K_M and K_i values for each substrate were determined. For K_i measurements, substrate was held constant at levels below the apparent K_M and rates determined in the presence of free SUMO paralogs as inhibitors. Data was fitted to the following equation to estimate kinetic constants.

$$V_0 = V_{\text{Max}} [S] / ([S] + K_M (1 + I/K_i)) \quad \text{Equation 1}$$

For K_M measurements minor adjustments had to be made for the fact that there was a small amount of SUMO present in the substrate (~10%). Thus, data was fitted to the following equation where $F=0.10$.

$$V_0 = V_{\text{Max}} [S](1-F) / ([S](1-F) + K_M (1 + F[S]/K_i)) \quad \text{Equation 2}$$

Irreversible reaction of vinyl sulfone reagents with endogenous SENPs and recombinant purified cSENP

Varying quantities of HA-SUMO1-VS or HA-SUMO2-VS (5ng–15ng) were incubated with lysates or recombinant proteins at 25°C for 15 min. The reaction was terminated by adding SDS sample buffer and boiling for 5 minutes. The samples were separated by 12% SDS-PAGE and visualized by Sypro and Imperial protein staining or transferred to a nitrocellulose membrane for western blotting.

Reactions were monitored using Covance mouse anti-HA.11 monoclonal in western blocking buffer (1x TBS, 0.05% Tween 20, 5% non-fat milk). Polyclonal antibodies against SENPs were raised in rabbit and affinity purified on immobilized antigen [4, 23]. Each SENP was detected in immunoblots using anti-SEN1 (full-length protein), anti-SEN2 (full-length protein), anti-SEN3 (residues 204–406, [4, 23]), anti-SEN5 (residues 1–237, [4, 23]), anti-SEN6, residues 1–499, [4, 23]) and anti-SEN7 (full-length protein) were used to detect each SENP. Secondary antibodies were HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG as required, and antibodies were detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

RESULTS

Most studies suggest that different SENP enzymes exhibit different affinities and varying degrees of selectivity for SUMO paralogs, although no comprehensive studies have examined all SENPs in a quantitative way. To compare and understand differences between SUMO proteases, we synthesized HA-tagged SUMO-VS [4, 23, 46, 47] and SUMO-AMC. These reagents are specific for deSUMOylating enzymes [44] and thus appropriate to be used in quantitative studies to characterize the catalytic capability and specificity of human

full-length SENPs and their catalytic domains acting on SUMO1 and SUMO2. We first examined the catalytic capability of the core catalytic domains to ascertain the range of possible substrates. Since these studies were carried out in the absence of other cellular components and on proteins that lack the physiological N-terminal domain, we also characterized the full-length endogenous SENPs in mammalian tissues and cell lysates.

Irreversible labeling of the catalytic domains of SENPs with HA-SUMO-VS

Seven human SENPs 1, 2, 3, 5, 6 and 7 have been identified in genomic databases and structural analysis has revealed that they are cysteine proteases. They all contain a conserved C-terminal catalytic domain and a variable N-terminal domain. (Figure 1). The non-conserved N-terminal regions are thought to determine subcellular localization and paralog specificity. In addition, SENP6 and SENP7 have small sequence inserts in their catalytic domain.

To determine the SUMO paralog preferences of the catalytic domain of SENPs, we expressed cSENP1, 2, 5, 6 and 7 in *E. coli* with an N-terminal His₆-tag and purified them on Ni²⁺ NTA agarose. Purified cSENPs were incubated with HA-SUMO1-VS or HA-SUMO2-VS and reactivity was monitored by following adduct formation using SDS-PAGE and protein staining (Figure 2A–E). Both cSENP1 (Figure 2A) and cSENP2 (Figure 2B) were efficiently labeled with SUMO1-VS and SUMO2-VS, suggesting that they recognize and bind SUMO and are potentially active with both SUMO1 and SUMO2. While cSENPs 5–7 (Figure 2C–E) react with both SUMO paralogs, they show incomplete reactivity as evidenced by residual unlabeled protein, and thus, in comparison with other cSENPs, SENP6 and 7 show low reactivity. At higher levels of SUMO-VS almost complete reaction is observed with all cSENPs, suggesting that the proteins are fully folded but weakly reactive with these reagents (data not shown). The purity of the HA-SUMO1-VS and HA-SUMO2-VS reagents is shown in supplementary Figure 1.

As reported previously [16], expression of cSENP3 in *E. coli* is poor and results in an unstable protein preparation (data not shown). Thus, we could not test the SUMO-VS reaction with this protein using direct protein staining. As a result, we verified adduct formation by western blotting of a lysate from *E. coli* cells expressing Flag-tagged cSENP3. A fraction of cSENP3 formed adducts with each SUMO-VS reagent (Figure 2F top). The higher molecular weight bands detected in the presence of SUMO-VS are reactive with anti-Flag antibody but did not form when the catalytic cysteine was mutated to alanine (Figure 2F bottom).

Thus, these data demonstrate that cSENP1, 2, 3, 5, 6 and 7 all can form adducts with SUMO1-VS and SUMO2-VS but there is little evidence of specificity among the SUMO paralogs. However, labeling with SUMO-VS reagents is irreversible and rapid and the above results reflect a combination of binding and catalytic capability rather than kinetic preferences.

Steady-state kinetics of SUMO-AMC hydrolysis by the cSENPs

To separately determine the binding and catalytic preferences of cSENPs, we used SUMO-AMC substrates and measured various catalytic parameters (Table 1 and Supplementary Figure 2). Catalytic rates (k_{cat}) vary about 100 fold with the fastest rate being 10^{-2} sec^{-1} . Catalytic efficiency (k_{cat}/K_M) varies 1,000 fold with the fastest rate ($2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) approaching the protein:protein diffusion limit. cSENP1 exhibits K_M values of 15nM for SUMO1-AMC and 40nM for SUMO2-AMC. while, the K_M values for cSENP2 are in the range of 20–30nM. Thus, cSENP1 and cSENP2 exhibit similar kinetic affinities for both SUMO paralogs. As shown in Figure 3A and Figure 3B, the ratio of paralog-specific k_{cat}

(maximal rate of catalysis) and k_{cat}/K_M (catalytic efficiency) indicate that cSEN1 and cSEN2 have no marked catalytic preference for either SUMO paralog.

In the case of cSEN5 and cSEN6, the K_M values for SUMO1-AMC are in the range of 150–180 nM, while the K_M values for SUMO2-AMC are 50–100 nM. These data reflect a similar kinetic affinity of cSEN5 and cSEN6 for SUMO1 and SUMO2 (Table 1). The ratio of paralog specific kinetic constants for cSEN5 and cSEN6 indicate a marked catalytic preference for SUMO2 (Figure 3A and Figure 3B). k_{cat}/K_M measurements show that cSEN5 and cSEN6 are approximately 30-fold more selective for SUMO2 than SUMO1. The activity of cSEN7 on SUMO1-AMC was undetectable, suggesting SEN7 cannot catalyze hydrolysis of SUMO1.

Binding of SUMO to cSENPs

To unambiguously determine the binding constants for each SUMO paralog we measured the K_i values for inhibition of SUMO-AMC cleavage by free SUMO paralogs. We determined inhibition constants by conducting kinetic studies in the presence of variable amounts of SUMO1 or SUMO2 (Table 2). With the exception of cSEN7, all cSENPs bound both substrates with affinities (K_i values) between 10–100 nM. The K_i/K_M ratios for cSENPs 1–2 and 5–6 are close to 1, both for SUMO1 and SUMO2. This suggests that catalysis is the rate-limiting step for these SENPs. The only exception was cSEN7, where both the K_i value and the K_i/K_M ratio are at least 10-fold higher than the other cSENPs. In general, K_i is a direct measure of the binding constant for free enzyme binding the product, SUMO, whereas K_M is a complex kinetic term that represents the concentration of substrate necessary to achieve half maximal concentration of the species preceding the rate-limiting step. The finding of a K_i/K_M ratio of ten suggests that either AMC contributes to the strength of substrate binding or that a step subsequent to formation of the acyl enzyme and release of AMC is rate limiting for SEN7 (see discussion).

SUMO paralog specificity of cellular SENPs

To characterize the paralog specificity of full-length SENPs in cell lysates we utilized SUMO-VS. The specificity of these reagents was confirmed using a well-characterized yeast protease, Ulp1, known to deSUMOylate proteins modified by both SUMO1 and Smt3 [48]. High molecular weight adducts resulted from the covalent modification of the active cysteine on Ulp1 with both SUMO-VS paralogs (Supplementary Figure 3). To confirm that this reaction is SUMO-specific, we performed this experiment with similar derivatives of ubiquitin and the ubiquitin-like proteins HA-Nedd8-VS and HA-ISG15-VS. Adducts were formed only in the presence of SUMO1-VS or SUMO2-VS (Supplementary Figure 3), confirming that these reagents are target specific and react with Ulp1. The catalytic domain of Ulp1 (His₆-Ulp1_{403–621}) also reacts with both SUMO1-VS and SUMO2-VS (data not shown). In separate experiments, Ubiquitin-VS, Nedd8-VS and ISG15-VS were tested with their respective optimal enzymes Uch13, Den1 and Ubp43 and were found to be fully functional [25, 44, 49]. Thus, in accordance with these results and those of others [4, 23, 42, 46, 47] reactivity with SUMO-VS preferentially (probably exclusively) detects SUMO-specific enzymes.

We next treated a HEK293 cell lysate (30 μg) with increasing amounts of HA-SUMO2-VS and monitored SENP3 adduct formation using SENP3 antibodies (Figure 4A). A gel shift is observed with increasing amounts of HA-SUMO2-VS. Complete conversion of free SENP3 to an adduct confirms that all detectable endogenous SENP3 reacts with SUMO2-VS. We performed similar experiments using SENP5 antibody with similar results (Figure 4B). We can estimate the amount of SENPs present in a lysate by noting the minimum amount of SUMO-VS necessary to consume all the free SENPs (Figure 4A and B). Based on the

minimum amount of SUMO-VS required to react with all the SENPs in these titrations, and an average molecular weight of the SUMO-VS adducts formed of $\sim 10^5$ daltons, we estimate that no more than about 0.06% of the lysate protein are SENPs.

The selectivity of cellular SENPs was compared by incubating HeLa cell lysates with either HA-SUMO1-VS or HA-SUMO2-VS and used specific polyclonal antibodies prepared against each full-length protease to determine the pattern of adduct formation for each endogenously expressed SENP (Figure 4C–H). Endogenous SENPs 2, 3, 5, 6 and 7 show preferential reactivity with SUMO2. In contrast, endogenous SENP1 forms higher molecular weight adducts with both SUMO1-VS and SUMO2-VS (Figure 4C). Multiple bands are detected with most of these antibodies and we do not know if all of these bands represent the respective SENPs. However, we can say that at least some of these bands are shifted and that this likely indicates the specificity of the indicated SENP. Unshifted bands may be other cross-reactive proteins or may represent catalytically inactive protein due to partial denaturation or covalent modification.

As expected, cSENP1 (Figure 2A) and endogenous full-length SENP1 (Figure 4C) form adducts with HA-SUMO1-VS and HA-SUMO2-VS. cSENP2 reacts with HA-SUMO1-VS (Figure 2B), but endogenous SENP2 does not (Figure 4D). The main difference between cSENP and endogenous SENP is that the endogenous protein contains the N-terminal domain and any potential post-translational modifications. In general, the N-terminal domain is projected to play regulatory roles *in vivo*, including contributions to substrate specificity and protein localization [15, 29].

To explore if the N-terminal domain of SENP2 modulates its catalytic specificity, we expressed full-length His₆-SENP1 and full-length His₆-SENP2 in *E. coli*. Homogenates were incubated with HA-SUMO1-VS or HA-SUMO2-VS, and adduct formation was confirmed by immunoblotting with anti-HA antibody. Similar to the pattern with endogenous proteins, His₆-SENP1 reacts with both SUMO1-VS and SUMO2-VS (Figure 5) whereas His₆-SENP2 reacts only weakly with SUMO1-VS (Figure 5). Thus, full-length recombinant His₆-SENP2 exhibits paralog selectivity similar to endogenous SENP2 suggesting that the N-terminal domain may contribute to substrate specificity. The presence of bands at molecular weights lower than full-length His₆-SENP1 and 2 suggests limited proteolysis of the recombinant proteins. We were unable to do detailed kinetic studies with full-length SENP1 and SENP2 due to the instability of these protein preparations (data not shown).

Labeling of SENPs in mammalian cells and tissue extracts

We also tested formation of adducts between SUMO-VS and SENPs in lysates of other cells to compare the specificity of deSUMOylation to that revealed by the kinetic measurements and the labeling experiments with HeLa cells (Figure 4).

We examined endogenous SENP selectivity by testing lysates from a panel of mammalian cells: HeLa, Cos7, HEK293, LMTK[−] and A9. Proteins labeled with the HA-SUMO-VS reagents were detected by western blotting with anti-HA antibody (Figure 6). We found that most cell lines appear to form an adduct of approximately 90–100kDa with SUMO1-VS. Given the specificity of cSENPs and the apparent molecular weight of the observed adduct, it is likely that this band represents SENP1 labeled irreversibly with SUMO1-VS. However, based on molecular weight alone this band could also correspond to SENP2 or SENP5. In the case of SUMO2-VS we detected corresponding adducts of various molecular weights indicating the possible labeling of SENP2, 3, 5, 6 or 7 (Figure 6). In the HeLa cell lysate labeled with SUMO2-VS, these shifted bands correspond to SENPs 1, 2, 3, 5, 6 and 7 when probed with SENP antibodies respectively (Figure 4C–H).

Finally we prepared tissue extracts from rabbit thymus, spleen, heart, brain, adrenal and thyroid glands to examine the distribution of active SENPs using the SUMO-VS reagents (Figure 7 and data not shown). Anti-HA antibody was used to analyze SENP labeling patterns as described above. The thymus in particular contains a significant amount of SENP proteases that form adducts with SUMO2-VS (Figure 7, lane 3 and Supplementary Figure 4). SUMO2-VS adducts are also observed in the spleen and adrenal gland lysates. However, the SENP protein levels in these lysates appear to be low and yield low levels of adducts, particularly for the SUMO1-VS reagent. Little significant SENP labeling was seen in brain or heart (data not shown). Rabbit tissue extracts could not be probed with the available anti-SENP antibodies, as they were all raised in rabbits.

Catalytic capability of tissues and cell lysates

To examine the balance of cellular activities acting upon SUMO1 and SUMO2, we measured the ability of these endogenous SENPs present in cell and tissue lysates to hydrolyze SUMO1-AMC or SUMO2-AMC (Table 3). We first characterized the SUMO1-AMC and SUMO2-AMC substrates by incubating with a panel of enzymes with selectivity for different ubiquitin-like proteins; GST-Ulp1 and His₆-Ulp1₄₀₃₋₆₂₁ (SUMO specific), Uchl3 (Ubiquitin selective), Den1 (Nedd8 selective) and Ubp43 (ISG15 specific). Hydrolysis was detected by an increase in fluorescence due to the release of AMC. As expected, SUMO1-AMC and SUMO2-AMC were only hydrolyzed by GST-Ulp1 and His₆-Ulp1₄₀₃₋₆₂₁ and not by ubiquitin-, Nedd8-, or ISG15-specific enzymes (data not shown) demonstrating that these substrates can be used to specifically detect deSUMOylating enzyme activity in crude extracts.

The levels of SUMO1-AMC and SUMO2-AMC hydrolysis seen in cell and tissue lysates (Table 3) suggest that most contain active SENPs capable of hydrolyzing SUMO-AMC. Consistent with the adduct formation data, SUMO2-AMC is hydrolyzed more readily (12-20 fold faster) than SUMO1-AMC, indicating that SUMO2 modified substrates are more likely to be processed in cells and tissues. Thymus and spleen are the richest source of SENPs, while brain and heart exhibit very low levels. This may suggest that deSUMOylation is more important in proliferating cells.

DISCUSSION

SENP1 is unique in its propensity to act efficiently on both SUMO1 and SUMO2

Previous biochemical studies suggest that SENP1 is a major SENP for processing and deconjugating SUMO1 [50] and is the most common SUMO-endoropeptidase in the cell [16, 21]. It forms adducts with SUMO1-VS [23, 46] and the residues responsible for the SUMO1-SENP1 association are known [20, 50, 51]. We confirmed that endogenous SENPs in cell lysates react with SUMO-VS giving at least one adduct with an approximate molecular weight of 100kDa (Figure 6), consistent with an irreversible association of SUMO1-VS with SENP1. We verified this result using SENP1 antibodies in HeLa lysates, and confirmed our hypothesis that endogenous SENP1 can associate with both SUMO1-VS and SUMO2-VS (Figure 4C).

A detailed study of the kinetics, structure and molecular mechanisms of the SENP1 catalytic domain suggests that it has a high affinity for SUMO and physiologically relevant substrates (RanGAP1-SUMO1), and lesser affinities for YFP-SUMO-CFP fusion proteins (SUMO1-FL and SUMO2-FL) [50]. We also observed high affinities for SUMO binding to cSENP1, with there being a slight preference for binding to SUMO1 (Table 2). The absolute values for binding of SUMO by cSENP1 measured by ITC [50] or kinetic inhibition (Table 2) agree to within an order of magnitude. Thus, there is little discrimination by cSENP1 for

SUMO paralogs with non-physiological leaving groups based on their binding preferences. Kinetic values of $K_M=15\text{nM}$ for SUMO1-AMC and $K_M=40\text{nM}$ for SUMO2-AMC are slightly lower than those reported previously for SUMO paralogs bearing non-physiological leaving groups [50]. The apparent K_M for cSEN1 catalyzed hydrolysis of SUMO1-FL and SUMO2-FL substrates is 98nM and 126nM respectively, values that are similar to the binding constants measured by ITC. The similarity of K_i and K_M values for non physiological substrates argues that the rate limiting step for these substrates is acyl enzyme formation. With a physiological substrate, SUMO1-RanGAP1, cSEN1 exhibits a K_M that is 15-fold higher than the binding affinity [50] arguing that release of RanGAP1 is much slower and at least partially rate-limiting. This is consistent with the presence of weak interactions between RanGAP1 and both SUMO1 and cSEN1 observed in the crystal structure of the cSEN1(C603A)-RanGAP1-SUMO1 [50].

The catalytic rate (k_{cat}) for different paralogs differs only two-fold for SUMO-AMC and SUMO-RanGAP1, but is 50-fold faster for cSEN1 acting on SUMO1-FL compared to SUMO2-FL. These differences are likely due to differences in substrate structure and orientation of the scissile bond. We find that when the leaving group is AMC, cSEN1 shows very similar kinetics for the hydrolysis reaction of either SUMO1 or SUMO2, as indicated by k_{cat}/K_M ratios and k_{cat} values (Table 1). We also observe slower k_{cat} rates than those observed previously for cSEN1 acting on SUMO1-FL [50]. This discrepancy may be due to suboptimal geometry at the scissile bond [52], the nature of the AMC group, or differences in the assay formats. We note however, that k_{cat}/K_M values reported for SUMO-RanGAP1 substrates are in excess of $10^9 \text{ M}^{-1} \text{ s}^{-1}$, values that greatly exceed the diffusion limit. It remains to be seen if those values can be confirmed. Nonetheless, our data, and that of others, clearly suggests that full-length SEN1 is a major endogenous SENP, and has a unique capability of effectively working on both SUMO1 and SUMO2 conjugates (Figure 4C and Figure 5A) not shared by other SENPs.

Prior studies indicate that the major organ for SEN1 protein expression is in the testis with detectable levels in thymus, pancreas, spleen, liver, ovary and small intestine and does not vary greatly for other SENPs [21]. We observed little adduct formation in tissue extracts after incubation with SUMO1-VS (Figure 7) suggesting that levels of SUMO1-specific SENPs are relatively low in tissues. This is confirmed by measurements of the rates of hydrolysis of SUMO1 and SUMO2-AMC in tissues and cell extracts (Table 3 and Supplementary Figure 4). Labeling with SUMO2-VS in rabbit thymus lysates show a 100kDa band that may be indicative of SEN1 or SEN2 (by molecular weight). The lower band of approximately 70kDa seen in thymus, spleen and adrenal tissue may be non-specific association with the VS reagents or a splice variant of an unidentified SENP.

SEN2 requires its N-terminal domain to regulate SUMO paralog specificity

cSEN2 reacted with SUMO1-VS while endogenous SEN2 in cell lysates reacted only weakly. SUMO-VS labeling experiments using lysates suggest that the N-terminal domain of SEN2 may exert a significant degree of regulation over the catalytic domain (Figure 4D). Nonetheless, we tested the ability of recombinant full-length SEN1 and SEN2 to react with SUMO1-VS or SUMO2-VS. As seen with endogenous SEN1 and SEN2 (Figure 4C and 4D), recombinant full-length SEN2 irreversibly reacts readily with SUMO2-VS, but only weakly with SUMO1-VS, while SEN1 reacts with both (Figure 5B and 5A).

Here, cSEN1, cSEN2 and full length SEN1 do not show a preference for SUMO paralogs in labeling with SUMO-VS or hydrolysis of SUMO-AMC. But full length SEN2, differing from cSEN2 only in the presence of the N-terminal sequence, exhibits a clear paralog specificity for SUMO2 over SUMO1. cSEN2 catalyzes hydrolysis of SUMO1-

AMC and SUMO2-AMC with K_M values of 30nM and 20nM respectively and similar k_{cat} values (Table 1). Previous studies have shown similar k_{cat} for both SUMO paralogs, but their apparent K_M was lower for RanGAP1-SUMO2 than for RanGAP1-SUMO1.[52]. Again, this discrepancy may be due to the nature of the AMC group, or differences in the assay formats. Full-length recombinant protein containing the N-terminal sequence is somewhat unstable and is not appropriate for detailed kinetic measurements (data not shown). The N-terminal domain of SENP2 has nuclear localization signals (NLS) and a nuclear export signal (NES) that facilitates movements between nucleus and cytoplasm [39]. In addition, the N-terminal domain of SENP2 is responsible for associating with the nuclear pore [32, 33], but it has not been directly implicated in specificity for different SUMO paralogs. Since SENP1 and SENP2 have differences in the N-terminus, they may also display differences in localization and substrate binding. Therefore, the N-terminal domains of SENPs could play an important role in direct recognition of substrates either by restricting or allowing access to the catalytic domain or by the action of scaffolds or adaptors for colocalization.

SENP3 and 5 show a strong preference for SUMO2

cSENP3 and endogenous SENP3 bound SUMO2-VS irreversibly, but not SUMO1-VS, indicating a very specific recognition (Figure 2F, Figure 4A and 4E). SENP3 is abundant in HeLa, in *Xenopus laevis* extracts, in thymus, and spleen tissues in human [4, 47]. Since it is well established that SENP3 binds SUMO2 modified nucleophosmin through the N-terminal region and displays specificity for SUMO2 and SUMO3 conjugates [4, 40, 43], it is likely that SENP3 plays a role in SUMO2/3 metabolism. Though we did not obtain detailed catalytic measurements for cSENP3, we detected a similar pattern of selectivity to that observed for cSENP5.

Figure 2C shows cSENP5 reacts with SUMO1-VS and Table 2 indicates a K_M =180nM for SUMO1-AMC. Nevertheless, Figure 4F suggests that endogenous SENP5 only reacts with SUMO2-VS and not SUMO1-VS. Because cSENP5 exhibits a K_M of 50nM for SUMO2-AMC, it appears that SENP5 is more likely to process SUMO2/3 than SUMO1 as suggested previously [22, 42]. Similarly, k_{cat}/K_M measurements revealed cSENP5 to be 10-fold more active on SUMO2 than SUMO1 (Figure 3, TABLE 2).

These data do not negate the possibility that SENP5 may process SUMO1 *in vivo*, in particular because the catalytic domain of SENP5 catalyzes AMC hydrolysis from SUMO1 with a k_{cat} that is only 4-fold slower than cSENP1 (Table 1) and it has been reported to deconjugate SUMO1 from PML [22]. Additionally, an increase in the levels of SUMO1 is observed when SENP3 and SENP5 are co-depleted from the cell [4] suggesting that these enzymes may play an indirect role in SUMO1 metabolism yet to be determined. One can speculate that if the enzyme were localized near the substrate through the actions of an adapter or scaffold it has sufficient catalytic capability to act upon that substrate. Further study is required to investigate the roles for SENP5 regarding SUMO1 deconjugation and processing.

The SENP6 and 7 family

Interestingly, both the SENP6 catalytic domain and endogenous SENP6 have a high catalytic selectivity for SUMO2 and specific reaction with SUMO2-VS (Figures 1D and 3G). We found that SENP6 has a K_M of 145nM and 100nM for SUMO1-AMC and SUMO2-AMC respectively. Thus, SENP6 may be mostly involved in SUMO2/3 pathway regulation especially in light of evidence that it preferentially acts on substrates containing three or more SUMO2/3 moieties [23]. Both cSENP6 and cSENP7 have higher isopeptidase

activity; cleave SUMO2 from RanGAP1 but not SUMO1 [16, 24, 53] and preferentially catalyze di-SUMO2 and poly-SUMO2/3 deconjugation [23, 24, 53].

Though our cSEN7 protein preparation was of somewhat lower yield [16] we were still able to perform kinetic analyses and determined a K_M of 80nM for SUMO2-AMC. cSEN7 did not hydrolyze SUMO1-AMC (Table 1) but reacted irreversibly with SUMO1-VS (Figure 2E). Apparently, the cSEN7-SUMO1 association is of low affinity and the leaving group in SUMO1-AMC may not be accessible for hydrolysis. We find that cSEN7 reacts weakly with both SUMO-VS derivatives forming the respective adducts, but endogenous SENP7 reacts only with SUMO2-VS (Figure 4H). These data are indicative of a role for SENP7 in SUMO2 related pathways. However, caveats include that cSEN7 may require the N-terminal domain to function properly and that specificity or stability may depend on post-translational modifications. The absence of the N-terminal α -helix in the cSEN7 crystal structure (that is present in SENP1 and SENP2 structures) may also relate to its unique function [53].

SENP Specificity and Catalysis

Catalysis is an inherent property of the SENP catalytic domain as indicated by kinetic data outlined in Table 1. All cSENPs, with the exception of cSEN7, catalyzed both SUMO1-AMC and SUMO2-AMC hydrolysis with K_M values in the nanomolar range. Specificity was evident in the kinetic studies of all the cSENPs; cSEN1 has a higher activity on SUMO1 and cSENPs 2, 5, 6 and 7 have higher activity on SUMO2 (Table 2). These preferences are largely due to differences in catalytic efficiencies and not the ability to bind either SUMO paralog. Thus, all cSENPs reacted with both SUMO1-VS and SUMO2-VS (Figure 2) and with the exception of SENP7 their affinity for SUMO paralogs varied narrowly between 20 and 200 nM. On the other hand, catalytic efficiencies varied by a thousand fold (10^3 to 10^6 $M^{-1}s^{-1}$). Interestingly, cSEN2 and cSEN5 have the highest k_{cat} (0.02 to 0.08 s^{-1} , Table 1), which may indicate that these enzymes have the highest rates in the cell. However, the absolute value of catalytic efficiency and rates may not be predictive of physiological function. Rather, they should be considered indicative of the potential catalytic activity if the enzymes actually encounter substrates, especially considering that these values are measured with catalytic fragments *in vitro*.

Specificity is likely achieved as a result of modulating the rates of acyl enzyme formation and release of the leaving group. SENPs catalyze a Ping Pong Bi Bi mechanism with water as the second substrate. At constant concentrations of water this becomes an Ordered Uni Bi mechanism where S=SUMO-AMC, P=AMC and Q=SUMO, and E'Q is the acyl enzyme [54].

Under initial rate conditions, P approaches zero and k_2 (acylation of the SENP with release of AMC) becomes the first irreversible step.

For this model, $K_M = \frac{k_{+3}k_{+4}(k_{-1}+k_2)}{k_1[k_3(k_2+k_4)+k_2(k_{-3}+k_4)]}$ and $k_{cat}/K_M = k_1k_2/(k_{-1}+k_2)$

When $K_i=K_M$ for SUMO, as is the case for SENP1, 2, 5 and 6, the simplest explanation is that $k_1/k_{-1}=k_{-4}/k_4$; i.e., the influence of the AMC moiety on substrate binding is minimal and acylation (k_2) is the rate limiting step. The identity of the SUMO paralog and the

leaving group would both be expected to strongly influence this rate, imparting specificity to catalysis.

When $K_i \gg K_M$ there are two options; either the presence of a leaving group (AMC in our case) is enhancing substrate binding ($k_1/k_{-1} \ll k_{-4}/k_4$) or the equilibrium for substrate binding is pulled by additional downstream equilibria preceeding a rate limiting step subsequent to acylation [54]. The subsequent steps involve hydrolysis of the acyl enzyme and release of SUMO and this step is identical regardless of the identity of the leaving group. The net rate at which the acyl enzyme is hydrolyzed and SUMO released is $k_3k_4/(k_{-3}+k_4)$. To distinguish between these mechanistic alternatives requires measurement of the corresponding parameters using substrates with alternative leaving groups. Previous studies showed that SENP7 has a preference for cleavage of SUMO2 derivatives [7, 55–58]. This same study provides estimates of the catalytic efficiency of SENP7 catalyzing the hydrolysis of di-SUMO2 ($\sim 0.2 \text{ s}^{-1}$), SUMO2-RanGAP1 (0.02 s^{-1}) and SUMO2 pro-protein ($< 10^{-4} \text{ s}^{-1}$). We find a rate for SUMO2-AMC hydrolysis of $2 \times 10^{-4} \text{ s}^{-1}$, consistent with these measurements. However, these studies make it clear that the rate of deacylation and SUMO release must exceed 0.2 s^{-1} and cannot be rate limiting in our studies. Thus, it is likely that the AMC leaving group is making favorable interactions with the substrate binding site and is bound much more tightly than is free SUMO2. It is possible that the additional sequence interrupting the core catalytic domain is contributing to this interaction with AMC and reflects additional interaction surfaces used in recognizing polySUMO2/3.

SUMO2/3 metabolism is more dynamic than that of SUMO1 in lysates

When we performed labeling experiments with SUMO-VS using cell lysates, we observed a higher concentration of proteins that form adducts with SUMO2-VS than with SUMO1-VS (Figure 4C–H). Based on the molecular weight shifts observed upon reaction with SUMO-VS and SENP antibodies (Figure 4A–4H), we believe that endogenous SENP2, 3, 5, 6 and 7 in cells and tissues preferentially react with SUMO2-VS. SUMO1/2-AMC substrates were both efficiently hydrolyzed by mammalian cell lysates and rabbit tissue extracts (Table 3). However, in all tissues and cell lysates examined there was a 15–20 fold higher activity on SUMO2-AMC than activity on SUMO1-AMC (Table 3). Thus, by both criteria there is much more capacity for SUMO2 metabolism than for SUMO1.

The total amount of SUMO2/3 in the cell is greater than that of SUMO1 [12], and the rapidity of response to stress [7, 55–58] implies that there is a dynamic equilibrium between SUMOylation and deSUMOylation processes. Depletion of SENPs 1, 2, 3, 5, 6 and 7 causes a dramatic increase in higher molecular weight conjugates [4, 23, 24, 42, 43]. These observations suggest a higher degree of SUMO2/3 dynamics in the cellular environment supporting an active role for SENP enzymes in regulatory processes.

The present systematic analysis of mammalian SENPs 1, 2, 3, 5, 6 and 7 supports a strong preference for SUMO2/3 relative to SUMO1. Further, our studies clearly indicate a role for the divergent N-terminus of SENPs in restricting activity on many SUMO substrates *in vivo*. The mechanisms of this regulation is most likely through localization, physical interaction with a SUMO modified substrate, or binding to an adapter that delivers and/or helps re-orient the substrate cleavage site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Dasso M. Emerging roles of the SUMO pathway in mitosis. *Cell Div.* 2008; 3:5. [PubMed: 18218095]
2. Seeler JS, Dejean A. Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol.* 2003; 4:690–699. [PubMed: 14506472]
3. Johnson ES. Protein modification by SUMO. *Annu Rev Biochem.* 2004; 73:355–382. [PubMed: 15189146]
4. Yun C, Wang Y, Mukhopadhyay D, Backlund P, Kolli N, Yergey A, Wilkinson KD, Dasso M. Nucleolar protein B23/nucleophosmin regulates the vertebrate SUMO pathway through SENP3 and SENP5 proteases. *J Cell Biol.* 2008; 183:589–595. [PubMed: 19015314]
5. Tatham MH, Jaffray E, Vaughan OA, Desterro JM, Botting CH, Naismith JH, Hay RT. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem.* 2001; 276:35368–35374. [PubMed: 11451954]
6. Bohren KM, Nadkarni V, Song JH, Gabbay KH, Owerbach D. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem.* 2004; 279:27233–27238. [PubMed: 15123604]
7. Vertegaal AC, Andersen JS, Ogg SC, Hay RT, Mann M, Lamond AI. Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. *Mol Cell Proteomics.* 2006; 5:2298–2310. [PubMed: 17000644]
8. Golebiowski F, Matic I, Tatham MH, Cole C, Yin Y, Nakamura A, Cox J, Barton GJ, Mann M, Hay RT. System-wide changes to SUMO modifications in response to heat shock. *Sci Signal.* 2009; 2:ra24. [PubMed: 19471022]
9. Matic I, van Hagen M, Schimmel J, Macek B, Ogg SC, Tatham MH, Hay RT, Lamond AI, Mann M, Vertegaal AC. In vivo identification of human small ubiquitinlike modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. *Mol Cell Proteomics.* 2008; 7:132–144. [PubMed: 17938407]
10. Schimmel J, Larsen KM, Matic I, van Hagen M, Cox J, Mann M, Andersen JS, Vertegaal AC. The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. *Mol Cell Proteomics.* 2008; 7:2107–2122. [PubMed: 18565875]
11. Blomster HA, Hietakangas V, Wu J, Kouvonen P, Hautaniemi S, Sistonen L. Novel proteomics strategy brings insight into the prevalence of SUMO-2 target sites. *Mol Cell Proteomics.* 2009; 8:1382–1390. [PubMed: 19240082]
12. Saitoh H, Hinchey J. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem.* 2000; 275:6252–6258. [PubMed: 10692421]
13. Azuma Y, Arnautov A, Dasso M. SUMO-2/3 regulates topoisomerase II in mitosis. *J Cell Biol.* 2003; 163:477–487. [PubMed: 14597774]
14. Rodriguez MS, Dargemont C, Hay RT. SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem.* 2001; 276:12654–12659. [PubMed: 11124955]
15. Ayaydin F, Dasso M. Distinct in vivo dynamics of vertebrate SUMO paralogs. *Mol Biol Cell.* 2004; 15:5208–5218. [PubMed: 15456902]
16. Mikolajczyk J, Drag M, Bekes M, Cao JT, Ronai Z, Salvesen GS. Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. *J Biol Chem.* 2007; 282:26217–26224. [PubMed: 17591783]
17. Mukhopadhyay D, Dasso M. Modification in reverse: the SUMO proteases. *Trends Biochem Sci.* 2007; 32:286–295. [PubMed: 17499995]
18. Drag M, Salvesen GS. DeSUMOylating enzymes--SENPs. *IUBMB Life.* 2008; 60:734–742. [PubMed: 18666185]

19. Hay RT. SUMO-specific proteases: a twist in the tail. *Trends Cell Biol.* 2007; 17:370–376. [PubMed: 17768054]
20. Shen LN, Dong C, Liu H, Naismith JH, Hay RT. The structure of SENP1-SUMO-2 complex suggests a structural basis for discrimination between SUMO paralogues during processing. *Biochem J.* 2006; 397:279–288. [PubMed: 16553580]
21. Xu Z, Au SW. Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1. *Biochem J.* 2005; 386:325–330. [PubMed: 15487983]
22. Gong L, Yeh ET. Characterization of a family of nucleolar SUMO-specific proteases with preference for SUMO-2 or SUMO-3. *J Biol Chem.* 2006; 281:15869–15877. [PubMed: 16608850]
23. Mukhopadhyay D, Ayaydin F, Kolli N, Tan SH, Anan T, Kametaka A, Azuma Y, Wilkinson KD, Dasso M. SUSP1 antagonizes formation of highly SUMO2/3-conjugated species. *J Cell Biol.* 2006; 174:939–949. [PubMed: 17000875]
24. Shen LN, Geoffroy MC, Jaffray EG, Hay RT. Characterization of SENP7, a SUMO-2/3-specific isopeptidase. *Biochem J.* 2009; 421:223–230. [PubMed: 19392659]
25. Gan-Erdene T, Nagamalleswari K, Yin L, Wu K, Pan ZQ, Wilkinson KD. Identification and characterization of DEN1, a deneddylase of the ULP family. *J Biol Chem.* 2003; 278:28892–28900. [PubMed: 12759362]
26. Wu K, Yamoah K, Dolios G, Gan-Erdene T, Tan P, Chen A, Lee CG, Wei N, Wilkinson KD, Wang R, Pan ZQ. DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *J Biol Chem.* 2003; 278:28882–28891. [PubMed: 12759363]
27. Reverter D, Lima CD. Insights into E3 ligase activity revealed by a SUMORanGAP1-Ubc9-Nup358 complex. *Nature.* 2005; 435:687–692. [PubMed: 15931224]
28. Mendoza HM, Shen LN, Botting C, Lewis A, Chen J, Ink B, Hay RT. NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J Biol Chem.* 2003; 278:25637–25643. [PubMed: 12730221]
29. Bailey D, O'Hare P. Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1. *J Biol Chem.* 2004; 279:692–703. [PubMed: 14563852]
30. Yeh ET, Gong L, Kamitani T. Ubiquitin-like proteins: new wines in new bottles. *Gene.* 2000; 248:1–14. [PubMed: 10806345]
31. Bailey D, O'Hare P. Herpes simplex virus 1 ICP0 co-localizes with a SUMO-specific protease. *J Gen Virol.* 2002; 83:2951–2964. [PubMed: 12466471]
32. Hang J, Dasso M. Association of the human SUMO-1 protease SENP2 with the nuclear pore. *J Biol Chem.* 2002; 277:19961–19966. [PubMed: 11896061]
33. Zhang H, Saitoh H, Matunis MJ. Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. *Mol Cell Biol.* 2002; 22:6498–6508. [PubMed: 12192048]
34. Nishida T, Kaneko F, Kitagawa M, Yasuda H. Characterization of a novel mammalian SUMO-1/Smt3-specific isopeptidase, a homologue of rat axam, which is an axin-binding protein promoting beta-catenin degradation. *J Biol Chem.* 2001; 276:39060–39066. [PubMed: 11489887]
35. Best JL, Ganiatsas S, Agarwal S, Changou A, Salomoni P, Shirihai O, Meluh PB, Pandolfi PP, Zon LI. SUMO-1 protease-1 regulates gene transcription through PML. *Mol Cell.* 2002; 10:843–855. [PubMed: 12419228]
36. Nishida T, Tanaka H, Yasuda H. A novel mammalian Smt3-specific isopeptidase 1 (SMT3IP1) localized in the nucleolus at interphase. *Eur J Biochem.* 2000; 267:6423–6427. [PubMed: 11029585]
37. Cheng J, Bawa T, Lee P, Gong L, Yeh ET. Role of desumoylation in the development of prostate cancer. *Neoplasia.* 2006; 8:667–676. [PubMed: 16925949]
38. Kim YH, Sung KS, Lee SJ, Kim YO, Choi CY, Kim Y. Desumoylation of homeodomain-interacting protein kinase 2 (HIPK2) through the cytoplasmic-nuclear shuttling of the SUMO-specific protease SENP1. *FEBS Lett.* 2005; 579:6272–6278. [PubMed: 16253240]
39. Itahana Y, Yeh ET, Zhang Y. Nucleocytoplasmic shuttling modulates activity and ubiquitination-dependent turnover of SUMO-specific protease 2. *Mol Cell Biol.* 2006; 26:4675–4689. [PubMed: 16738331]

40. Nishida T, Yamada Y. SMT3IP1, a nucleolar SUMO-specific protease, deconjugates SUMO-2 from nucleolar and cytoplasmic nucleophosmin. *Biochem Biophys Res Commun.* 2008; 374:382–387. [PubMed: 18639523]
41. Cheng J, Perkins ND, Yeh ET. Differential regulation of c-Jun-dependent transcription by SUMO-specific proteases. *J Biol Chem.* 2005; 280:14492–14498. [PubMed: 15701643]
42. Di Bacco A, Ouyang J, Lee HY, Catic A, Ploegh H, Gill G. The SUMO-specific protease SENP5 is required for cell division. *Mol Cell Biol.* 2006; 26:4489–4498. [PubMed: 16738315]
43. Haendl M, Harasim T, Eick D, Muller S. The nucleolar SUMO-specific protease SENP3 reverses SUMO modification of nucleophosmin and is required for rRNA processing. *EMBO Rep.* 2008; 9:273–279. [PubMed: 18259216]
44. Wilkinson KD, Gan-Erdene T, Kolli N. Derivatization of the C-terminus of ubiquitin and ubiquitin-like proteins using intein chemistry: methods and uses. *Methods Enzymol.* 2005; 399:37–51. [PubMed: 16338347]
45. Dang LC, Melandri FD, Stein RL. Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. *Biochemistry.* 1998; 37:1868–1879. [PubMed: 9485312]
46. Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, Kolli N, Gan-Erdene T, Wilkinson KD, Gill G, Lima CD, Ploegh HL, Ova H. Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol Cell Biol.* 2004; 24:84–95. [PubMed: 14673145]
47. Wang Y, Mukhopadhyay D, Mathew S, Hasebe T, Heimeier RA, Azuma Y, Kolli N, Shi YB, Wilkinson KD, Dasso M. Identification and developmental expression of *Xenopus laevis* SUMO proteases. *PLoS ONE.* 2009; 4:e8462. [PubMed: 20041154]
48. Li SJ, Hochstrasser M. A new protease required for cell-cycle progression in yeast. *Nature.* 1999; 398:246–251. [PubMed: 10094048]
49. Borodovsky A, Ova H, Kolli N, Gan-Erdene T, Wilkinson KD, Ploegh HL, Kessler BM. Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chem Biol.* 2002; 9:1149–1159. [PubMed: 12401499]
50. Shen L, Tatham MH, Dong C, Zagorska A, Naismith JH, Hay RT. SUMO protease SENP1 induces isomerization of the scissile peptide bond. *Nat Struct Mol Biol.* 2006; 13:1069–1077. [PubMed: 17099698]
51. Xu Z, Chau SF, Lam KH, Chan HY, Ng TB, Au SW. Crystal structure of the SENP1 mutant C603S-SUMO complex reveals the hydrolytic mechanism of SUMO-specific protease. *Biochem J.* 2006; 398:345–352. [PubMed: 16712526]
52. Reverter D, Lima CD. Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates. *Nat Struct Mol Biol.* 2006; 13:1060–1068. [PubMed: 17099700]
53. Lima CD, Reverter D. Structure of the human SENP7 catalytic domain and poly-SUMO deconjugation activities for SENP6 and SENP7. *J Biol Chem.* 2008; 283:32045–32055. [PubMed: 18799455]
54. Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I Nomenclature and rate equations. *Biochim Biophys Acta.* 1963; 67:104–137. [PubMed: 14021667]
55. Saracco SA, Miller MJ, Kurepa J, Vierstra RD. Genetic analysis of SUMOylation in *Arabidopsis*: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. *Plant Physiol.* 2007; 145:119–134. [PubMed: 17644626]
56. Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY, Vierstra RD. The small ubiquitin-like modifier (SUMO) protein modification system in *Arabidopsis*. Accumulation of SUMO1 and -2 conjugates is increased by stress. *J Biol Chem.* 2003; 278:6862–6872. [PubMed: 12482876]
57. Manza LL, Codreanu SG, Stamer SL, Smith DL, Wells KS, Roberts RL, Liebler DC. Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol.* 2004; 17:1706–1715. [PubMed: 15606148]

58. Huang C, Han Y, Wang Y, Sun X, Yan S, Yeh ET, Chen Y, Cang H, Li H, Shi G, Cheng J, Tang X, Yi J. SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *EMBO J.* 2009; 28:2748–2762. [PubMed: 19680224]

Figure 1. Human SENP proteases

The white bars represent nonconserved N-terminal regions. The conserved catalytic domain is shown in grey. Note the insertions in the catalytic domains of SENPs 6 and 7 shown in black. Green circle indicates SUMO-binding Motifs.

Figure 2. Irreversible labeling of the catalytic domains of SENPs with HA-SUMO-VS
A–E) Recombinant cSENPs (cS) 1, 2, 5, 6, and 7 were reacted with HA-SUMO1-VS and HA-SUMO2-VS for 15 minutes at 25°C. Samples were run on 12% SDS-PAGE and visualized by Imperial protein stain. Asterisks show adduct formation with all cSENPs indicates reactivity with SUMO1-VS and SUMO2-VS. F) FLAG-cSEN3WT and FLAG-cSEN3C-A (active site mutation) were reacted with HA-SUMO1-VS and SUMO2-VS for 15 minutes at 25°C. The reactions were monitored by immunoblotting using anti-FLAG antibody. FLAG-cSEN3 WT formed adduct with both SUMO1-VS and SUMO2-VS, whereas FLAG-cSEN3CA did not form adducts. Asterisks indicate the vinylsulfone adducts.

Figure 3. Kinetic constants for cSENPs catalyzed hydrolysis of SUMO-AMC Summary of steady state kinetic constants for the hydrolysis of SUMO1-AMC and SUMO2-AMC by the catalytic domains of human SENPs

A) (k_{cat}/K_M) ratio, SUMO2/SUMO1. B) k_{cat} ratio, SUMO2/SUMO1

Figure 4. Paralog preference of endogenous SENPs

A–B) HEK293 lysate (30 μ g) was titrated with HA-SUMO2-VS and reacted for 15 minutes. Adduct formation was monitored by 12% SDS-PAGE and western blotting with anti-SENP3 and SENP5 antibodies in separate experiments. Lanes 5–6 of panel A indicates that all endogenous SENP3 in the lysate reacted with SUMO2-VS. Similarly in Figure 4B lanes 5–6 show that all endogenous SENP5 reacted with SUMO2-VS. C–H) HeLa cell lysate was incubated with HA-SUMO1-VS or HA-SUMO2-VS for 15 minutes. The incorporation of SUMO-VS into SUMO-specific proteases was monitored by 12% SDS-PAGE and immunoblotting with anti-SENPs 1, 2, 3, 5, 6 and 7 antibodies.

Figure 5. N-terminus of SENP1 and SENP2 contributes to SUMO paralog specificity
 Full-length His₆-SENP1 and His₆-SENP2 recombinant proteins, purified by nickel resin, were labeled with HA-SUMO1 or HA-SUMO2-VS. Reactions were terminated with sample buffer and analyzed by western blotting with anti-HA antibody. Asterisks indicate probable degradation products.

Figure 6. SUMO2 specific SENPs are more abundant than SUMO1 specific SENPs in mammalian cell lysates

HA-SUMO1-VS or HA-SUMO2-VS (5ng) were reacted with HEK293, HeLa, LM TK⁻, A9 and Cos7 cell lysates. Reaction mixtures were analyzed by 12% SDS PAGE and western blotting with Anti-HA antibody. The expected migration for each adduct is indicated by the arrows.

Figure 7. Detection of SENPs in rabbit tissue extracts

Rabbit tissue extracts were prepared and incubated with HA-SUMO1-VS or HA-SUMO2-VS. Reactions were terminated and resolved by 12% SDS-PAGE and probed with anti-HA antibody. No SUMO-VS was added in the first lane in each panel. The arrows mark the positions of the observed SUMO-VS adducts. The asterisk marks non-specific covalent labeling with SUMO-VS.

TABLE 1

Steady-state kinetic analysis of cSENPs for SUMO-AMC substrates

	k_{cat} (s^{-1})		K_M (nM)		k_{cat}/K_M $M^{-1} s^{-1}$		Correlation coeff. (R^2)	
	1AMC	2AMC	1AMC	2AMC	1AMC	2AMC	1AMC	2AMC
cSENPI	3.9×10^{-2}	2.4×10^{-2}	15.9	42.5	2.4×10^6	5.6×10^5	0.95	0.96
cSENPII	5.9×10^{-3}	1.9×10^{-3}	32.8	19.7	1.8×10^5	9.9×10^4	0.95	0.97
cSENPIV	1.1×10^{-2}	8.4×10^{-2}	176	52.3	6.1×10^4	1.6×10^6	0.95	0.90
cSENPIV	3.0×10^{-4}	9.5×10^{-3}	145	97.4	2.1×10^3	9.8×10^4	0.95	0.96
cSENPIV	-	2.1×10^{-4}	-	80.2	-	2.6×10^3	-	0.98

Kinetic parameters of cSENPs catalytic activity (K_M , k_{cat} and k_{cat}/K_M values) were determined using SUMO-AMC hydrolysis assay for each substrate (Dang and Melandri, 1998). Experiments were performed in triplicate and the resulting data were fit to a Michaelis and Menten equation.

Table 2

Binding constants of cSENPs for SUMO paralogs

	K_i (nM)		K_i/K_M (nM)		Correlation coefficient (R ²)	
	1AMC	2AMC	1AMC	2AMC	1AMC	2AMC
cSENPI	23.8	90.8	1.5	2.1	0.96	0.98
cSENPII	12.9	36.7	0.4	1.9	0.98	0.94
cSENPIII	45.5	47.9	0.3	0.9	0.97	0.95
cSENPIV	94.7	56.4	0.6	0.6	0.90	0.98
cSENPIV	-	860.0	-	10.7	-	0.91

The affinity of cSENPs for SUMO paralogs was determined by measuring the k_i for competitive inhibition of hydrolysis of SUMOI-AMC and SUMO2-AMC by the addition of free SUMOI or SUMO2.

TABLE 3
Catalytic Activity of endogenous SENPs in mammalian cell lysates and tissue extracts by SUMO-AMC substrate hydrolysis

	SUMO1-AMC (units/mg)	SUMO2-AMC (units/mg)	SUMO2/SUMO1
Rabbit Tissue extracts			
Thymus	0.11 ± .01	1.6 ± .2	14.5
Spleen	0.06 ± .007	1.0 ± .16	16.6
Adrenal gland	0.04 ± .003	0.61 ± .12	15.2
Thyroid gland	0.02 ± .001	0.24 ± .04	12.0
Brain	-	0.1 ± .01	-
Heart	-	0.02 ± .003	-
Mammalian Cell lysates			
LM TK ⁻	0.05 ± .004	0.71 ± .09	14.2
A9	0.02 ± .003	0.41 ± .005	20.5
NIH3T3	0.02 ± .004	0.41 ± .01	20.5

The specific activity of endogenous SENPS present in mammalian cell lysates and rabbit tissue extracts to hydrolyze SUMO1-AMC or SUMO2-AMC substrates was determined spectrophotometrically (Dang and Melandri, 1998).