The Dynamics and Mechanism of SUMO Chain Deconjugation by SUMO-specific Proteases*

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Miklós Békés†§, John Prudden¶, Tharan Srikumar‖, Brian Raught¶, Michael N. Boddy¶, and Guy S. Salvesen‡§

From the †Sanford-Burnham Medical Research Institute, La Jolla, California 92037, the §Molecular Pathology Graduate Program, University of California, San Diego, La Jolla, California 92037, the ¶Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and the ‖Ontario Cancer Institute and McLaughlin Centre for Molecular Medicine, MaRS Centre, Toronto M5G 1L7, Canada

SUMOylation of proteins is a cyclic process that requires both conjugation and deconjugation of SUMO moieties. Besides modification by a single SUMO, SUMO chains have also been observed, yet the dynamics of SUMO conjugation/deconjugation remain poorly understood. Using a non-deconjugatable form of SUMO we demonstrate the underappreciated existence of SUMO chains in vivo, we highlight the importance of SUMO deconjugation, and we demonstrate the highly dynamic nature of the SUMO system. We show that SUMO-specific proteases (SEPNs) play a crucial role in the dynamics of SUMO chains in vivo by constant deconjugation. Preventing deSUMOylation in Schizosaccharomyces pombe results in slow growth and a sensitivity to replication stress, highlighting the biological requirement for deSUMOylation dynamics. Furthermore, we present the mechanism of SUMO chain deconjugation by SENPs, which occurs via a stochastic mechanism, resulting in cleavage anywhere within a chain. Our results offer mechanistic insights into the workings of deSUMOylating proteases and highlight their importance in the homeostasis of (poly)SUMO-modified substrates.

Reversible post-translational modification of proteins by ubiquitin-like covalent modifiers is a widely utilized mechanism to alter the fate, binding partners, function, or localization of a given target protein (1). A prime example is the covalent attachment of multiple ubiquitin moieties to a lysine side-chain of a target protein (2), leading to the formation of ubiquitin chains, which have different functions depending on the lysine utilized in the internal ubiquitin linkage. Recently, small ubiquitin-like modifier (SUMO)² has also been shown to form chains in vitro (3, 4) and in vivo (5, 6).

The SUMO conjugation pathway consists of SUMO activation, transfer, and ligation enzyme machinery analogous to the ubiquitin pathway (7–9). Somewhere upwards of 500 cellular proteins are SUMOylated in vivo (10), yet the extent of observable SUMOylation is only a snapshot due to the presence of SUMO-specific proteases, SENPs. The SUMO cycle begins and ends with specific proteolytic events: the processing of polySUMO and the deconjugation of SUMO from the target protein by SENPs (11). In addition to monoSUMOylation, there is now growing evidence that SUMO, like ubiquitin, forms polymeric chains. Thus, the non-covalent interaction of the SUMO conjugating enzyme, Ubc9, with SUMO has presented a possible mechanism for SUMO chain formation (4, 12) and indeed in vivo, all SUMO molecules (Smt3, the Saccharomyces cerevisiae SUMO homologue, and human SUMO1, -2, and -3) have been observed to form SUMO chains, primarily via lysine residues located near their N termini (13). In vitro the SUMOylation system is quite permissive, allowing for multiple SUMO-SUMO linkages (14). The initial indication that SUMO chains exist in vivo came from transfection experiments yielding multimers of SUMO2 conjugated to HDAC4 (3) and from S. cerevisiae (15). More recently, evidence for in vivo SUMO chains comes from mass-spectrometry experiments using HeLa cells, revealing chain formation via Lys-11 of SUMO2 and SUMO3 (5). However, the extent of polySUMO versus monoSUMO modification of the proteome has yet to be established, although polySUMOylation has been reported to increase during heat shock treatments (10). Once SUMO chains have been assembled on a substrate they could have two fates. They can either be disassembled by SENPs, presumably recycling the SUMO monomers and the target protein (11, 16) or, at least in theory, delivered to the proteasome via SUMO-targeted ubiquitin E3 ligases (STUB1s) that cause the ubiquitin-mediated degradation of polySUMO-modified substrates (17).

SEPNs are cysteine proteases that belong to peptidase clan C48 and share a common fold of their catalytic domain (18). In S. cerevisiae there are two deSUMOylating enzymes, Ulp1 and Ulp2, while humans have six (SENP1, -2, -3, -5, -6, and -7). SENPs are dual function proteases that can remove the C-terminal extensions from SUMO precursors and deconjugate SUMO from modified proteins (19). They exhibit substantial substrate specificity (20, 21) and a distinction has been established that sets SENP6 and SENP7 apart from other human SENPs, largely because of their poor ability to process SUMO precursors and monoSUMOylated RanGAP1. Rather, SENP6 and SENP7 have been proposed to be chain-specific, partly because SENP6 and SENP7 are most similar to yeast Ulp2,
which has been shown to limit the amount Smt3 chains in yeast (15).

Several questions remain to be answered; how are SUMO chains recognized and deconjugated by SENPs, how dynamic is the process, and how important are the dynamics? To answer these questions we utilized two human SENPs, SENP1, and SENP6, that diverge substantially in specificity and sequence conservation to determine the mechanism of SUMO chain deconjugation. Furthermore, by using a conjugatable, but not deconjugatable version of SUMO, which targets physiological substrates, we investigated how SENPs participate in and regulate the dynamics of SUMO chains in vivo. We also investigated the influence of irreversible SUMOylation on cellular growth rate and sensitivity to replication stress in Schizosaccharomyces pombe.

**EXPERIMENTAL PROCEDURES**

**LC-MS/MS of SUMO2 Chain Expressing E. coli Lysates**—For LC-MS/MS analysis, 4 mg of SENP-treated and untreated E. coli lysates were treated with 6 M guanidine and 10 mM DTT, alkylated with 30 mM iodoacetamide, and buffer exchanged into 8 M urea, 50 mM HEPES, pH 7.4, TCA precipitated, and re-dissolved in 50 mM HEPES, pH 8.0. Samples were desalted on C$_{18}$-ZipTip columns, re-dissolved in 0.1% trifluoroacetic acid and 2% acetonitrile and 3 μg of material was loaded onto an Orbitrap-XL mass spectrometer (Thermo) via a C18-column and eluted with 3 M final concentration and cleaved with a 2-fold serial dilution of 1 μM SENPs for 30 min at 37 °C, then visualized by SDS-PAGE and Coomassie staining. All constructs were verified to have the correct orientation of cleavable (wild-type) or non-cleavable (Q90P) tri-SUMO2 moieties by DNA sequencing as well as Western blotting for FLAG and for His following ± SENP-treatment of the purified proteins (see supplemental Fig. S7).

**Cell Culture, Transfection, Lysate Preparation, and Purification**—HEK293A and HeLa cells were readily maintained in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM l-glutamine. Full-length and catalytic-domain SENP constructs and all the SUMO variants were transfected into cells using NanoJuice (Novagen) according to the manufacturer’s protocol. 6–48 h post-transfection cells were washed twice with cold PBS and lysed on ice in 50 mM HEPES, pH 7.4, 150 mM KCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% CHAPS, supplemented with the protease inhibitors trans-epoxysuccinyl-l-leucylamido(4guanidino) butane, 3,4-dichloroisocoumarin, leupeptin and MG-132 in the presence or absence of 30 mM of the cysteine modifier N-ethylmaleimide (NEM, Pierce). To purify FLAG-tagged SENPs from HEK293A cells, lysates were incubated overnight at 4 °C with anti-FLAG agarose-beads (M2, Sigma), then washed three times in lysis buffer, and twice in PBS and eluted with 3×-FLAG-peptide (Sigma) and labeled with HA-SUMO2 vinylmethyl ester for 30min at 37 °C to ensure their activity. Lysates were used immediately for subsequent experiments or kept at −20 °C.

**Knockdown of SUMO2 and SUMO3 by siRNA**—A pool of five individual siRNA oligonucleotides (Qiagen) that target the 3’-UTR region of the SUMO2 and the SUMO3 mRNA were used in the experiments at 3 nM final concentration per individual siRNA (see supplemental methods for sequences used). HEK293A cells were reverse-transfected with the siRNA alone or together with rescue plasmids according to the manufacturer’s protocol and the cells were harvested 48 h post-transfections and the lysates were probed with the indicated antibodies. Phase-contrast images of cells were taken with a camera attached to an Olympus IX71 microscope (Olympus) at ×100 and ×200 magnification (data not shown). For growth curve experiments HEK293A cells were reverse-transfected at low confluence in 6-well plates and immediately transferred to 24-well plates, split six-ways and maintained in full DMEM for the indicated times, then trypsinized and counted manually using a hemocytometer by Trypan Blue (Sigma) staining.
**SENP-mediated SUMO Chain Dynamics**

*S. pombe* Experiments—pREP41-eGFP-pmt3-Q108P was cloned via standard PCR protocols using pmt3 cDNA and primers described in supplemental methods. The resulting product was then NdeI-BamHI digested and cloned into pREP41. Western blot analysis of total levels of SUMOylation has been described previously (23). The *S. pombe* strains used for this study are listed in supplemental Table S1.

**RESULTS**

The Dynamics of SUMO Chain Deconjugation by Endogenous SENPs—When cells are lysed under non-denaturing conditions (24) almost no SUMOylated proteins are observable. Including a cysteine modifier such as NEM or the denaturant SDS retains a certain level of SUMOylation, and the general conclusion is that endogenous constitutively active SENPs rapidly deconjugate SUMOylated proteins as a lysis artifact (24). We hypothesized that SENPs continuously deconjugate SUMO from its targets, and that a dynamic balance exists between SUMO addition and deconjugation, which is not represented in the instantaneous steady state captured in lysates. To address the dynamics of the SUMOylation cycle *in vivo*, we utilized a mutation within the cleavage site of SUMO that allows for conjugation, but is defective in deconjugation by SENPs (25).

We created eukaryotic expression constructs of wild-type (wt) mature SUMO2 and the aforementioned Q90P mutant, containing N-terminal epitope tags, and transfected them into HEK293A cells. The conjugating enzymes of the SUMO pathway tolerate the Q90P mutant because both the SUMO E1 and E2 have large active site cavities (9, 26) and can accommodate a slightly kinked SUMO C terminus introduced by Pro in P4, however the narrow active site cleft of SENPs does not tolerate it (27). Upon cell lysis, Q90P SUMO2 remains extensively conjugated in even in the absence of NEM (Fig. 1A), demonstrating that while the SUMO conjugation machinery accepts this mutant, endogenous SENPs are unable to remove it during lysis and presumably *in vivo* as well. The analogous mutation in SUMO1 also results in stabilized SUMOylation (supplemental Fig. S1), however, it does not significantly enhance SUMOylation over wt levels. We therefore focused our studies on SUMO2/3, which are also the physiologically relevant chain forming SUMO paralogs.

To further demonstrate the rapid dynamics of the SUMO pathway we performed a transfection time course experiment showing the expression of wild-type and the Q90P mutant SUMO2 (Fig. 1B). We begin to see both monomeric unconjugated and higher molecular weight (HMW) conjugated species of wild-type SUMO2 as early as six hours post-transfection. On the contrary, the Q90P mutant mainly appears as HMW conjugated species. The intermediates observed are consistent with variable length SUMO chains generated due to cleavage by SENPs. Importantly, a SUMO2 Q90P mutant, in which the three Lys residues (Lys-6, Lys-8, and Lys-11) in the N terminus were mutated to Arg to limit chain elongation (3), prevented the appearance of the multimeric cleavage intermediates.
Thus, by definition and logic, the universe of SUMOylated proteins contains abundant SUMO chains.

In control experiments we demonstrate that the HMW-conjugates are indeed polySUMO adducts, because they are cleaved by SENPs but not by a promiscuous deubiquitinating enzyme (data not shown). We ruled out the possibility that a putative endogenous *E. coli*-purified factor could be responsible for the deconjugation because a catalytically dead ∆N-SENP1 with an Ala replacing the active site Cys does not remove the HMW SUMO conjugates (data not shown) and we also show that SUMO chains of variable lengths from numerous gel-filtration fractions are collapsed in a similar fashion (supplemental Fig. S2). Notably, the N-terminal domain of SENP1 does not substantially influence cleavage of Q90P HMW-SUMO conjugates because full-length, wild type SENP1 also trims SUMO chains to SUMO multimers, showing that SUMO chain deconjugation is an intrinsic property of human SENPs (supplemental Fig. S3).

To ensure the validity of the SUMO2 Q90P mutant in "permanently" SUMOylating endogenous substrates, we looked for SUMO conjugates by mass spectrometry. We developed SUMO-PROTOMAP (supplemental Fig. S4), a method inspired by a technique developed by Dix et al. (28), which is capable of identifying SUMO conjugates by virtue of a downshift of a deSUMOylated substrate through deconjugation by SENP1 (see supplemental methods for details). In this small-scale study we were able to identify known SUMOylation substrates using SUMO2 Q90P (supplemental Fig. S5), confirming that this mutant SUMO stabilizes endogenous (poly)SUMO conjugates.

**Free and Substrate-anchored SUMO Chains Are Substrates of SENPs**—Having demonstrated the extensive existence of SUMO chains in vivo because of the Q90P mutant, we wanted to know the reason behind their presumed ephemeral existence. An obvious explanation is that they are such excellent substrates of SENPs that the cleavage intermediates are hard to demonstrate experimentally, because we only observe them using the Q90P mutant. To demonstrate that SENPs efficiently cleave SUMO chains, we purified SUMO chains expressed in *E. coli*, either as unanchored chains, or anchored to the canonical SUMO substrate RanGAP1, by employing a polycistronic expression vector encoding SUMO E1, E2, and SUMO2 (29). Unanchored SUMO2 chains could be collapsed entirely upon treatment by a deSUMOylating enzyme (Fig. 3A). Subjecting the total *E. coli* lysate expressing SUMO2 chains to LC-MS/MS after tryptic digestion, and filtering for specific SUMO conjugates (30) revealed that the primary SUMO-SUMO linkage is via Lys-11 of SUMO2. However, we were also able to detect a less abundant linkage via Lys-42 (Fig. 3B and supplemental Fig. S6).

The catalytic domains of both SENP1 and SENP6 (∆N-SENP1 and ∆N-SENP6), as examples of the different classes of SENPs (19), efficiently deconjugate SUMO2 chains produced in *E. coli*, whether or not they are attached to a heterologous
protein acceptor, such as RanGAP1 (Fig. 3, C and D). Interestingly, even though our recombinant enzymes are comparably reactive toward an activity-based probe, \( \text{H9004} \)N-SENP1 cleaves SUMO2 chains more efficiently than does \( \text{H9004} \)N-SENP6 (Fig. 3, C and D). We therefore conclude that SUMO chain deconjugation (at least \textit{in vitro}) is not a unique property of SENP6 and SENP7, but other SENPs share this activity.

Preliminary studies that we conducted with heterogeneous mixtures of multiple SUMO chains of various lengths did not allow us to determine whether SUMO chains are cleaved (i) from the free chain end, one SUMO at a time (processive deconjugation) or (ii) randomly anywhere within the chain (stochastic deconjugation) giving rise to various multimeric cleavage intermediates. We therefore developed an \textit{in vitro} model system to specifically address the mechanism of SUMO chain deconjugation.

**Linear SUMO Chains Are Cleaved in a Stochastic Manner**—We designed a set of linear tri-SUMO2 constructs mimicking isopeptide-linked chains to generate a homogenous system. In this system we can address the importance of each cleavage site within a chain individually. Because SUMO chains are predominantly linked by Lys-11 of SUMO2 and SUMO3 \textit{in vivo} (5, 31), we generated linear concatamers lacking the first 11 residues of SUMO2 to closely approximate this linkage. In these linear constructs, three \( \Delta N \)-SUMO2 molecules are fused “head-to-tail” (N to C terminus by a peptide bond) to simulate an isopeptide-linked tri-SUMO2 (Fig. 4A). Each concatamer has an N-terminal FLAG-tag and a C-terminal His tag that allows discrimination of the fragments after SENP-cleavage based on the size of the cleaved SUMO monomers (Fig. 4B) and by Western blotting (supplemental Fig. S7). Next, we used the cleavage prohibitive mutation Q90P to direct cleavage by SENPs at specific sites within linear chain (Fig. 4A). If all three available cleavage sites contain the Q90P mutation, the tri-SUMO2 construct is uncleavable (Fig. 4B and supplemental Fig. S8A) in the context of our linear constructs, where SENPs act as endopeptidases.

We defined \( EC_{50} \) as the amount of protease required to cleave 50% of the substrate in a given time. For those constructs containing only a single cleavage site, there was no significant difference in the \( EC_{50} \) values, irrespective of the position of the cleavage site within the constructs for either \( \Delta N \)-SENP1 (Fig. 4C) or \( \Delta N \)-SENP6 (Fig. 4D). Thus, there is no preferential recognition of any SUMO moiety within such a linear chain. This is further supported by allowing cleavage after both the first and second moiety, resulting in the simultaneous processing of the tri-SUMO2 construct from both ends, giving rise to the appearance of all three SUMO monomers (supplemental Fig. S8B). However, we noticed that cleavage after the last SUMO moiety, just before the His tag, slightly enhances cleavage (2-fold decrease in \( EC_{50} \)) for all the SENPs tested. This is consistent with their preference for shorter C-terminal fragments when assayed on proSUMOs (20).

Our data point to a stochastic mechanism for chain cleavage of our model tri-SUMO2 constructs, and reveal that both SENP1 and SENP6, as examples of different classes of SENPs, share this mechanism. Therefore, specificity is directed by the
primary recognition mechanism of a single SUMO moiety, at least at the level of the catalytic domain of SENPs. Full-length human SENP1 also does not discriminate between cleavage sites when assayed on linear triSUMO2 constructs (supplemental Fig. S9), indicating that mechanistically it is no different from its catalytic domain. Taken into account that full-length SENP1 also trims HMW-conjugates of SUMO Q90P (supplemental Fig. S3) it is safe to say that whatever the function of the N-terminal domain of SENP1 might be, it has no significant restrictive effects on the processing of SUMO chains.

Cellular Consequences of the Lack of SUMOylation/deSUMOylation—In the fission yeast S. pombe deletion of Pmt3 (the sole S. pombe SUMO homologue) displays a phenotype with an increased sensitivity to DNA damage (32). To address the biological role of deSUMOylation we generated Pmt3-Q108P to replicate the Q90P mutation of human SUMO2. GFP-tagged wild-type Pmt3 and the Q108P mutant were ectopically expressed in the pmt3/h9004 strain, and as expected, the Q108P mutant enhanced stable Pmt3-conjugates (Fig. 5A). Additionally, when plated on hydroxyurea, /H9004 Pmt3 cells expressing the Q108P mutant grew just as slowly as the deletion strain (Fig. 5B). This demonstrates that not only SUMOylation, but also deSUMOylation, is required for an efficient response to replicative stress. These findings are in line with the phenotype of S. cerevisiae lacking Ulp2, where this deSUMOylating protease is required for cell division following genotoxic stress (33).

FIGURE 4. Linear tri-SUMO2 constructs are cleaved in a stochastic manner by ΔN-SENP1 and by ΔN-SENP6. A, schematic representation of linear tri-SUMO2 constructs. Open green circles represent cleavable (WT) ΔN11-SUMO2 and full red circles represent uncleavable (Q90P) ΔN11-SUMO2. The lighting bolt indicates the cleavage site. B, purified tri-SUMO2 proteins were diluted to 1 μM and cleaved with 50 nM ΔN-SENP1 for 30 min at 37 °C and visualized by SDS-PAGE and Coomassie Blue staining. All tri-SUMO2 proteins are only cleaved at the allowed wild-type cleavage site (where P4 is Gln) and not when P4 is mutated to Pro (Q90P). In panels C and D the different tri-SUMO2 proteins (2 μM) were cleaved with 2-fold serial dilutions of 1 μM ΔN-SENP1 (C) or ΔN-SENP6 (D) for 30 min at 37 °C and visualized by SDS-PAGE and Coomassie Blue staining. The order of cleavage sites within tri-SUMO2 for each panel is indicated in the bottom right corner by the order of the red and green circles. E stands for lane with enzyme alone, and S stands for lane with substrate only. Panels in D have been cropped for clarity. The black triangles (▼) indicate approximate EC50 values.
To perform similar tests in human cells, we sought to deplete HEK293A cells of both SUMO2 and SUMO3 simultaneously and reconstitute them with wt SUMO and the Q90P mutants. Double knock-down of HEK293A cells reconstituted with the Q90P SUMO mutants exhibited slower growth, indicating that in the absence of endogenous SUMO-2/3 the non-deconjugatable mutant is disadvantageous for cellular growth (Fig. 5, C and D). Whereas it has previously been shown that SENPs are important in cell function, our results place the need for deSUMOylation and (by the definition and example through the Q90P mutant) the need for SUMO chain deconjugation in an evolutionary context, highlighting the extensive dynamics of (de)SUMOylation across lower and higher eukaryotes.

**DISCUSSION**

The Mechanism of SUMO Chain Deconjugation by SENPs—We demonstrate that SUMO chain cleavage by the catalytic domains of human SENP1 and SENP6 is a very rapid and stochastic event. Clearly we cannot rule out that SENPs not investigated in our study may have a processive ability, however we observed no mechanistic difference between the catalytic domain of SENP1 and SENP6 (the most divergent human SENPs). Therefore we propose that stochastic SUMO chain cleavage is a universal mechanism across the SENP family because of the common fold of these proteases. This should also apply to non-human deSUMOylating enzymes such as *S. cerevisiae* Ulp1, which is structurally similar to human SENPs (34), and has also been shown to cleave in vitro generated SUMO1 chains (35). It has been proposed that insertion loops in the catalytic domains of SENP6 and SENP7 could enhance their polySUMO specificity, however the deletion of the SENP7 loop did not impair its ability to cleave SUMO chains (36). Furthermore, the insertion loop of SENP6 is also dispensable for its activity, specificity and its chain cleaving mechanism (data not shown). This is in line with the results presented here that the core catalytic domain of SENPs alone, such as ΔN-SENP1, can catalyze SUMO chain deconjugation. Therefore, the difference between SENPs is not in terms of the mechanism of how they recognize polySUMO, but in terms of rates of cleavage of SUMO conjugates. It has also been recently suggested that the N-terminal domains of SENPs, which could contain putative SUMO-interacting motifs (SIMs), may confer a processive chain-cleaving specificity (37). However, we observed the same stochastic mechanism of chain cleavage using both full-length and catalytic domain only forms of SENP1, arguing against this proposal.

Our studies, conducted on linear triSUMO2 as a model of endogenous chains, point to a quite flexible arrangements of the SUMO moieties within the chain. This flexibility is reflected in modeling isopeptide-linked, natural SUMO2-SUMO2 link-
ages (Fig. 6), suggesting that the Δ11-SUMO2 trimer could be a faithful mimic of isopeptide-linked SUMO chains. Also, furthermore supporting a stochastic chain-cleaving mechanism of isopeptide-linked conjugates as well, is the capture of multi-meric cleavage intermediates when cleaving SUMO Q90P HMW-conjugates. Thus, the synthetic linear chains are valuable surrogates for natural SUMO polymers. As emerging crystal structures of various di-ubiquitin linkages have revealed different conformations of ubiquitin chains (38, 39), we eagerly await atomic level structural studies on di-SUMO molecules that would reveal the extent of flexibility of endogenous SUMO chains.

A unified stochastic mechanism of SUMO chain deconjugation for SENPs is in contrast to ubiquitin chain deconjugation by several DUBs, which have evolved different substrate recognition mechanisms and ancillary ubiquitin binding domains, giving rise to DUBs that prefer different ubiquitin linkages (40) or various length isopeptide-linked ubiquitin and others that cleave only mono-ubiquitin (41). Importantly, many DUBs, undergo significant conformational changes upon substrate binding (41–43), but SENPs show no such rearrangements within their catalytic domains (21, 44) and are presumed to be constitutively active.

SENP displays a great degree of specificity for the three human SUMO paralogs in terms of SUMO precursor activation and in terms of the deconjugation of monoSUMOylated RanGAPI, the canonical model substrate that has been used to assay the isopeptidase activity of SENPs (20, 21, 37). This specificity is largely based on contacts at the SUMO-SENP interface, with minimal conserved contacts elsewhere. However, there is a kinetic difference in the cleavage of peptide-linked versus isopeptide-linked SUMO moieties, such that SENP1 is a better isopeptidase than an endopeptidase for SUMO2 (20). The isopeptide bond between SUMO and its target is better positioned for cleavage than the equivalent peptide bond in proSUMO, as revealed in the crystal structures of the RanGAPI-SUMO2: SENP2 complex compared with proSUMO2:SENP2 (45). This supports our findings that the Q90P mutant in a linear construct (peptide-linked) is less sensitive to cleavage than in natural conjugates (isopeptide-linked). Thus, although we see absolutely no cleavage of the peptide-linked Q90P SUMO2, we observe low, but measurable, activity of SENP1 on isopeptide-linked Q90P SUMO2 HMW-conjugates.

**DeSUMOylation in Vivo**—The number of biological pathways that SUMOylation has been shown to be involved in is expanding (46) but it is not clear how important deSUMOylation is in these events. Our results, in which the phenotype of the ΔPmt3 S. pombe sensitivity to replication stress is maintained upon reconstitution with the Gln to Pro mutant, demonstrate the importance of deSUMOylation *in vivo*. Along this logic and by definition, the deSUMOylation of SUMO chains is just as important. Even though the Q90P mutant strongly suggests unbalanced SUMO chain formation, a biological function for SUMO chain formation is equivocal. An Smt3 mutant unable to form SUMO chains rescues some of the phenotypes caused by the deletion of the deSUMOylating enzyme Ulp2, with the conclusion that SUMO chain formation is not required for essen-
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S. cerevisiae (15), although SUMO chains clearly form in vivo.

In human cells, SENP6 depletion in HeLa cells leads to aberrant kinetochores (47), and interestingly, another human deSUMOylating enzyme, SENP5, has also been shown to be required for cell division (48), arguing for the importance of deSUMOylation in this regard. This is reflected by the slow growth phenotype of SUMO2/3-depleted cells reconstituted with the Q90P mutant, possibly through the inability to cleave SUMO chains on a global scale.

Genetic knock-outs or cellular knock-downs of deSUMOylating enzymes can highlight the requirement for the protein itself, but unless the knock-down is accompanied by reconstitution with a catalytic mutant (18, 47, 49) one cannot determine whether the catalytic activity or just the presence of the protein is required. The application of cleavage-resistant mutants takes an orthogonal approach in defining the importance of substrate cleavage on a more global scale, which would be cumbersome to achieve by one-by-one knock-down of SENPs. Cleavage-resistant mutants have been successfully deployed to define the role of specific proteolysis of proteins such as coagulation factor V in regulating coagulation (50), and for the retinoblastoma protein in uncoupling its tumor promoting function from its tumor suppressor one (51).

SUMO Q90P also fulfills this role because it demonstrates the importance of deSUMOylation on a global scale and allows us to define the mechanism of SUMO chain deconjugation. The inability of SENPs to remove SUMO Q90P leads to a massive shift in SUMOylation dynamics, and uncovers the prevalence of SUMO chains in vivo. Thus, our studies offer an important additional insight into the biology of human SENPs by highlighting their role as active negative regulators of SUMO chains, a feat that would have been difficult to achieve globally using genetic manipulation techniques.

The Dynamics of SUMO Chains—The intrinsic and rapid chain deconjugation activity of human SENPs renders SUMO chains a challenging phenomenon to observe and decipher. It is generally assumed that tight spatial and temporal regulation of encounter between SENPs and their substrates is required in vivo. For example, Ulp1 in S. cerevisiae is sequestered in the nucleolus following stress by low-levels of alcohol, allowing transient SUMO conjugate build-up (52). However, our results imply that a subset of SENPs constantly cleaves SUMOylated proteins and SUMO chains, essentially contributing an editing function that balances the ligation of SUMO onto its targets. This hypothesis is supported in principle by the finding that RanGAP1 is SUMOylated equally well by both SUMO1 and SUMO2, but in vivo it is conjugated to SUMO1, because the SUMO2-conjugated RanGAP1 is constantly deSUMOylated by SENPs (53).

Importantly, the multimeric cleavage intermediates only observed with the Q90P SUMO2 mutant reveal that a portion of HMW-conjugated species are modified by SUMO chains in vivo, and suggest that SUMO chains are such excellent substrates of SENPs that under normal experimental conditions they are difficult to detect. An alternative explanation for the lack of cleavage intermediates for wt SUMO2 is that the HMW conjugates observed are composed of proteins modified by multiple monoSUMOylation. However this does not preclude the possibility that during the life of the cell those proteins have also been modified by SUMO chains, but have been rapidly deconjugated by endogenous SENPs. It also follows that the majority of SUMO-conjugated proteins identified (10, 54, 55) may even represent the worst possible SENP substrates. These are the proteins that “escape” deSUMOylation and are detectable by various experimental conditions that can only prevent deSUMOylation upon cell lysis and not during the life of the cell.

The SUMO Q90P mutant identified physiologically relevant multi-SUMOylated substrates, and we suggest that it could also lead to the formation of free, unanchored SUMO chains, which could be a heterogeneous mixture of endogenous wild-type and transfected Q90P SUMO moieties. The idea of free SUMO chains having a signaling capacity has recently surfaced (56), however it has yet to be experimentally demonstrated. On the other hand, unanchored ubiquitin chains were recently shown to have a role in activating the kinase TAK1 (57). We suggest that an important function of SENPs is to monitor SUMO chain formation, whether conjugated or free chains, to ensure that polySUMO-interactors function appropriately (58, 59). An emerging concept in cell signaling proposes a fundamental role for tight regulation of ubiquitin chain editing, driven by the deconjugating proteases, in pathways such as NFKB (60) and death receptors signaling (61). Based on our observations, we propose a parallel function for the regulation of SUMO chain dynamics through SENPs in cell signaling. We also envision that the utility of conjugatable, but not deconjugatable ubiquitin-like modifiers will aid in tackling the physiological and pathological consequences of regulation by this highly versatile form of post-translational modification.

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