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Mechanism and Consequences for Paralog-Specific Sumoylation of Ubiquitin-Specific Protease 25

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SUMMARY

Vertebrates express two distinct families of SUMO proteins (SUMO1 and SUMO2/3) that serve distinct functions as posttranslational modifiers. Many proteins are modified specifically with SUMO1 or SUMO2/3, but the mechanisms for paralog selectivity are poorly understood. In a screen for SUMO2/3 binding proteins, we identified Ubiquitin Specific Protease 25 (USP25). USP25 turned out to also be a target for sumoylation, being more efficient with SUMO2/3. Sumoylation takes place within USP25's two ubiquitin interaction motifs (UIMs) that are required for efficient hydrolysis of ubiquitin chains. USP25 sumoylation impairs binding to and hydrolysis of ubiquitin chains. Both SUMO2/3-specific binding and sumoylation depend on a SUMO interaction motif (SIM/SBM). Seven amino acids in the SIM of USP25 are sufficient for SUMO2/3-specific binding and conjugation, even when taken out of structural context. One mechanism for paralog-specific sumoylation may, thus, involve SIM-dependent recruitment of SUMO1 or SUMO2/3 thioester-charged Ubc9 to targets.

INTRODUCTION

Posttranslational modification with ubiquitin and ubiquitin-like proteins (Ubl) of the SUMO family plays an important role in the regulation of cellular processes (Hershko and Ciechanover, 1998; Johnson, 2004; Hay, 2005; Kerscher et al., 2006; Geiss-Friedlander and Melchior, 2007). It involves isopeptide bond formation between the carboxyl group of the modifier and the ε-amino group of a lysine residue in the target. Ubls attachment requires a multistep enzymatic process: Ubls are first processed by specific proteases to expose their C-terminal diglycine motif. The mature Ubl is then transferred to the substrate via a cascade of E1 (activating), E2 (conjugating), and E3 (ligase) enzymes. The conjugation pathway for SUMO involves a single E1-activating enzyme (Aos1/Uba2) and a single conjugating enzyme (Ubc9) and is often facilitated by E3 enzymes (e.g., PIAS family and RanBP2). Reversibility of this process is ensured by a small family of SUMO isopeptidases (Mukhopadhyay and Dasso, 2007; Hay, 2007).

While lower eukaryotes (e.g., yeast, flies, and worms) express only one SUMO protein, vertebrates express at least three different SUMO paralogs: SUMO1, SUMO2, and SUMO3. Mature SUMO2 and SUMO3 (referred to as SUMO2/3) are 97% identical but differ substantially from SUMO1 (~50% identity). Conjugation of particular SUMO isoforms to their substrates in vivo is apparently highly specific and strictly regulated both temporally and spatially (Saitoh and Hinchey, 2000; Ayaydin and Dasso, 2004; Vertegaal et al., 2006). One clear example for preferential SUMO1 conjugation is RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997; Saitoh and Hinchey, 2000). However, in vitro conjugation of SUMO1 or SUMO2/3 using only the E1 (Aos1/Uba2) and E2 (Ubc9) enzymes shows no differences on RanGAP1 or Sp100 (Bossis et al., 2005; Tatham et al., 2005).

While SUMO E3 ligases and peptidases show some preference for individual SUMO family members (Reverter and Lima, 2004; Tatham et al., 2005), these do not suffice to account for the large number of paralog-specific SUMO targets. One mechanism that could contribute to paralog-specific sumoylation is the noncovalent interaction between SUMO proteins and their binding partners. So far, only one class of SUMO interacting motifs (SIM/SBM) has been described (Minty et al., 2000; Song et al., 2004; Hannich et al., 2005; Hecker et al., 2006), in contrast to many different ubiquitin-binding domains (Hicke et al., 2005; Hurley et al., 2006). The SIM contains a hydrophobic core and is often flanked by acidic or serine residues. As revealed by NMR studies, the hydrophobic core interacts with a groove of SUMO formed by a β sheet and part of the α helix (Song et al., 2004; Hecker et al., 2006). Interestingly, SIMs can interact in two orientations with SUMO depending on the position of the acidic stretch and whether the hydrophobic core is V/I-V/I-X-V/I or V/I-X-V/I-V/I. Only a few proteins have been identified to contain a functional SIM, and of these, most (e.g., p73α, PML, TDG, and Daxx) are known SUMO targets (Muller et al., 1998; Minty et al., 2000; Takahashi et al., 2005; Lin et al., 2006). Experiments on TDG and Daxx have implicated noncovalent interaction with SUMO for covalent conjugation with SUMO (for a discussion, see Hochstrasser, 2007). This would be related to recently discovered mechanisms of ubiquitylation that either involves recruitment of ubiquitylated E3 ligases or thioester-charged E2-conjugating enzymes to substrates with ubiquitin-binding domains (Woelk et al., 2006; Hoeller et al., 2007).

To identify factors that contribute to paralog-specific sumoylation, we carried out a biochemical screen for specific SUMO1- and SUMO3-binding proteins (G. Sauer and F.M., unpublished

data). A preferential SUMO2/3 binding protein was identified as ubiquitin specific protease 25 (USP25), a member of the deubiquitinating enzymes (DUBs). DUBs mediate the processing and the removal of ubiquitin. They can be divided into different classes, such as the cysteine proteases USP, UCH, OTU, MJD, and the metalloprotease family JAMM (Nijman et al., 2005). USP25, which belongs to the USP family, contains an ubiquitin-associated domain (UBA) and two ubiquitin interaction motifs (UIMs). Although several DUBs contain ubiquitin-binding domains, their role in DUB function is largely unknown (also see the Discussion).

Here we report that USP25 is a target for SUMO conjugation that is preferentially conjugated with SUMO2/3 as a consequence of its favorable binding to SUMO2/3, compared to SUMO1. We identify two sumoylation sites within the first and directly adjacent to the second ubiquitin interaction motif. These UIMs are required for the full catalytic potential of USP25 toward ubiquitin chains. Importantly, sumoylation of USP25 impairs its activity as a result of reduced affinity to ubiquitin chains.

RESULTS

Identification of USP25 as a Preferential SUMO2/3-Binding Protein

In a biochemical screen for proteins that bind specifically to SUMO1 or SUMO2/3, we identified USP25 as a SUMO2/3-interacting candidate. USP25 is an ~130 kDa protein that belongs to the family of deubiquitinating enzymes (DUBs). It contains one UBA and two UIM domains preceding the catalytic domain, as well as a predicted coiled-coil region (Figure 2A). USP25 is a vertebrate-specific DUB for which tissue-specific alternative splice variants have been observed; however, its biological function and regulation are not yet known (Valero et al., 2001; Bosch-Comas et al., 2006).

To verify that USP25 indeed interacts noncovalently with SUMO2/3, we transiently transfected HA-USP25 into HeLa cells and incubated the lysates with immobilized SUMO1, SUMO3, or control beads. As shown in Figure 1A, USP25 interacts more efficiently with SUMO3 compared to SUMO1. In order to study endogenous USP25, we raised polyclonal antibodies against full-length USP25. Using these antibodies, we also observed that endogenous USP25 interacts more efficiently with SUMO3 (Figure 1B). To examine whether USP25 directly interacts with SUMO or requires a bridging factor, we expressed and purified full-length untagged USP25 from bacteria (Figure 1C) and incubated it with immobilized SUMO1 or SUMO3. As shown in Figure 1D, USP25 indeed binds directly to SUMO and, again, more efficiently with SUMO3.

Characterization of the SUMO-USP25-Binding Interface

To define which residues of USP25 are involved in the interaction with SUMO, we generated a series of USP25 deletion mutants (Figure 2A). Binding studies with immobilized SUMO3 suggest that a region surrounding the first UIM (amino acids 91–119) is involved in the interaction with SUMO3 (Figure 2B). Closer inspection of this region uncovered a putative SUMO interaction motif. Mutagenesis of the bulky hydrophobic amino acids to alanine residues revealed that this region is indeed required for interaction with SUMO3 (Figure 2C) and for the weaker binding to

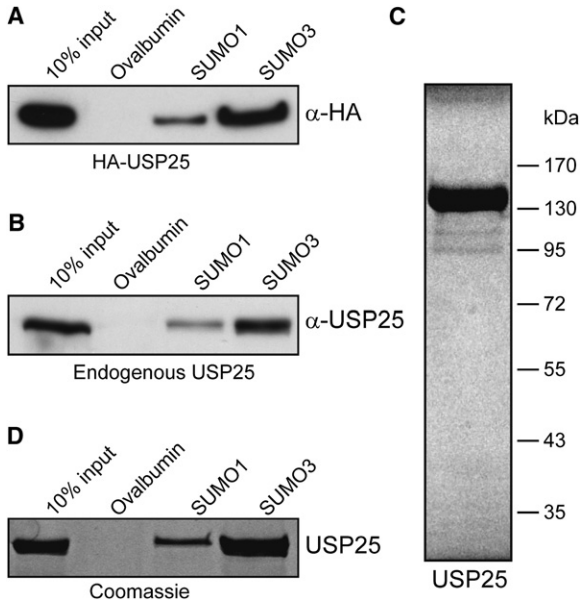

Figure 1. USP25 Interacts More Efficiently with SUMO2/3 Compared to SUMO1

(A) HeLa cells (one 5 cm dish per sample) were transfected with 5 μg HA-USP25. Upon lysis, cell extracts were incubated with 10 μg of immobilized Ovalbumin, SUMO1, or SUMO3. After extensive washing, bound USP25 was detected by immunoblotting using anti-HA antibodies.

(B) Cell extract from HeLa cells (one 10 cm plate per sample) was incubated as in (A) and endogenous USP25 was detected with anti-USP25 antibodies.

(C) Coomassie staining of purified recombinant USP25; molecular weight is depicted in kDa.

(D) Recombinant USP25 (10 μg) was incubated as in (A) and detected by Coomassie staining.

SUMO1 (data not shown). To provide further evidence that the USP25 SIM is required for binding to SUMO, we mutated residues in SUMO3 that are involved in SIM binding (Song et al., 2004). As shown in Figure 2D, single point mutations of SUMO3 already decreased binding with USP25, while the double mutant SUMO3 I32A-K33A was drastically impaired in USP25 binding. Taken together, these findings demonstrate that the interaction between USP25 and SUMO3 involves the SUMO-SIM interface.

USP25 Is a SIM-Dependent SUMO Target

Since several known SUMO targets can interact noncovalently with SUMO (Boddy et al., 1996; Muller et al., 1998; Minty et al., 2000; Takahashi et al., 2005; Lin et al., 2006), we tested whether USP25 is also a target for sumoylation. Thus, we carried out in vitro sumoylation reaction in the presence (Figure 3A) or absence of E3 ligases (Figures 3C and 3D). Indeed, USP25 can be efficiently conjugated with SUMO in vitro, even in the absence of an E3 ligase. When low Ubc9 concentrations were used, the reaction became E3 ligase-dependent (Figure 3A). Among the available enzymes, recombinant PIAS-Xα was most efficient. To demonstrate that USP25 can also be conjugated with SUMO in vivo, Flag-USP25 and HA-SUMO3 were transfected into HEK293T cells. Upon denaturing lysis, Flag-USP25 was immunopurified, and its sumoylation was analyzed by western

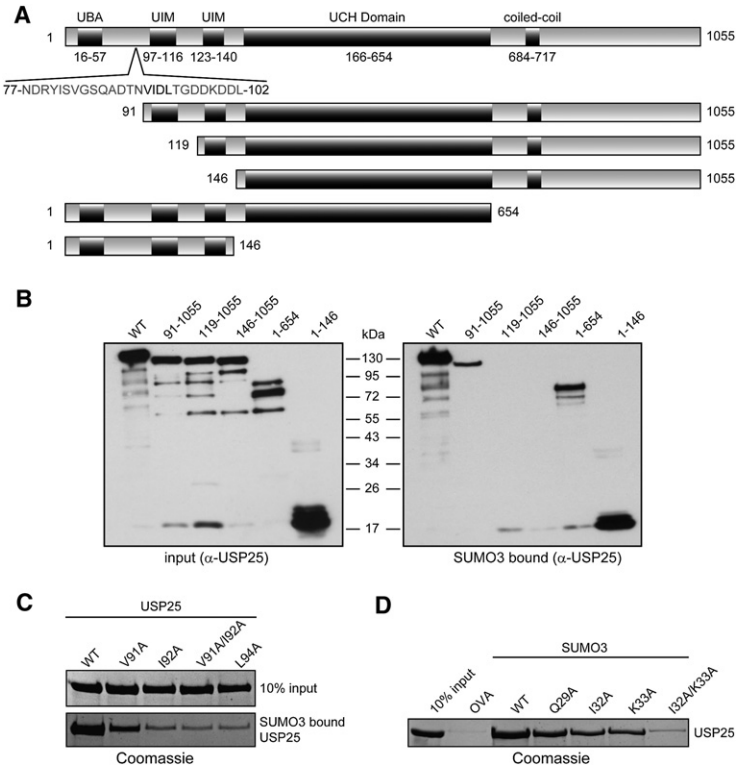

Figure 2. USP25 Binding to SUMO Requires a SIM in the N Terminus

(A) Schematic representation of USP25 deletion mutants.

(B) Recombinant deletion mutants of USP25 (1 μg) were incubated with 10 μg immobilized SUMO3 and washed, and bound proteins were detected by immunoblotting with anti-USP25 antibodies.

(C) Recombinant SIM mutants of USP25 (10 μg) were incubated as above and detected by Coomassie staining.

(D) Recombinant USP25 (10 μg) was incubated with different His-SUMO3 mutants immobilized to CnBr beads. USP25 was visualized by Coomassie staining.

blotting. The presence of slower migrating bands that are increased upon overexpression of Ubc9 provides strong evidence that USP25 can be sumoylated in vivo (Figure 3B, upper panel). The presence of multiple bands could be a consequence of sumoylation at multiple sites and/or SUMO2/3 chain formation.

We next addressed the question whether sumoylation of USP25 requires SIM-mediated interaction with SUMO. For this, we first tested in vitro SUMO conjugation of WT USP25 compared to USP25 SIM mutants in the absence of E3 ligases (Figure 3C). Indeed, the efficiency of sumoylation correlates with USP25's ability to bind noncovalently to SUMO (compare Figure 3C to Figure 2C). Of note, both WT USP25 and USP25 SIM mutants bind to Ubc9 in pull-down assays (Figure S1). High concentrations of free SUMO2/3 impaired conjugation efficiency (Figure S2), suggesting that free SUMO can compete with Ubc9-SUMO thioesters in USP25 recognition. To unequivocally prove that the SUMO-SIM interaction is a prerequisite for modification, we examined sumoylation of USP25 with the SUMO3 mutant I32A/K33A that is impaired in its noncovalent interaction with USP25 (see above, Figure 2D). As shown in Figure 3D, USP25 is not conjugated with this mutant. Of note, SUMO3 I32A/K33A forms Ubc9-thioesters efficiently (data not shown) and is conjugated to RanGAP1 like wild-type SUMO3

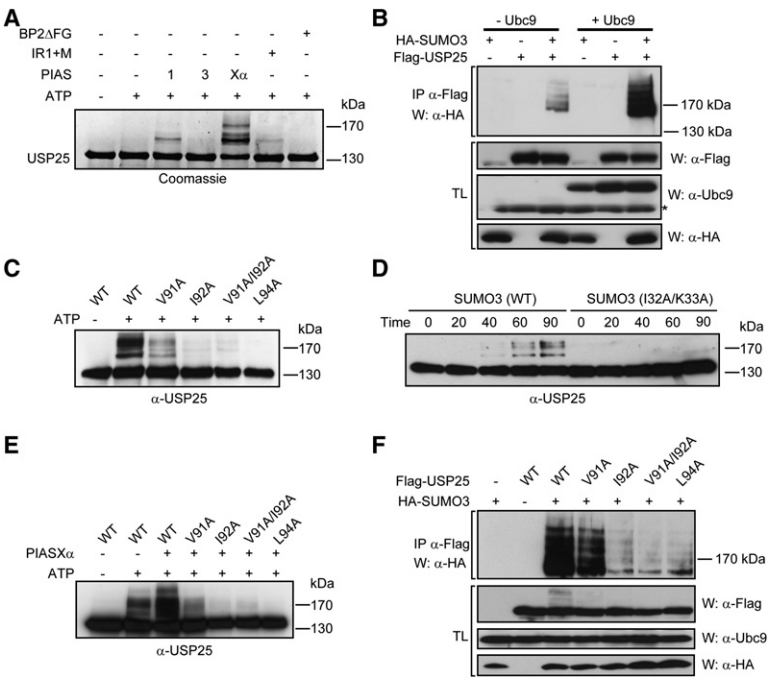

Figure 3. Sumoylation of USP25 Depends on Its SIM

(A) In vitro sumoylation of USP25 with various SUMO E3 ligases. USP25 (0.8 μM) was incubated for 1 hr at 30°C with 10 nM Aos1/Uba2, 50 nM Ubc9, and 100 nM of the depicted E3 ligase, 5 μM SUMO, and analyzed by Coomassie staining.

(B) HEK293T cells (one 10 cm dish per sample) were transfected with 5 μg Flag-USP25 and 5 μg HA-SUMO3 in the presence or absence of 1 μg Ubc9. Immunoprecipitations were performed with anti-Flag, after which the immunoprecipitates were analyzed by immunoblotting with anti-HA. Total lysates (TL) were analyzed with anti-Flag for USP25 expression, anti-Ubc9 for Ubc9 expression, and anti-HA for SUMO expression. The asterisk (*) indicates endogenous Ubc9, which migrates faster than transfected tagged-Ubc9.

(C) In vitro sumoylation reaction of USP25 SIM mutants (100 nM) in the presence of 10 nM E1 (Aos1/Uba2), 500nM E2 (Ubc9), 5 μM SUMO3, and 5mM ATP for 30 min at 30°C. Samples were analyzed by western blot with anti-USP25 antibodies.

(D) Time course of in vitro sumoylation of USP25 with His-SUMO3 and His-SUMO3 (I32A-K33A) as in (C). Time points are indicated in minutes.

(E) In vitro sumoylation of USP25 (100 nM) in the presence of 10 nM Aos1/Uba2, 40 nM Ubc9, 20 nM GST-PIASXα and 5 μM SUMO3.

(F) HEK293T cells were transfected with USP25 SIM mutants and analyzed as in (B).

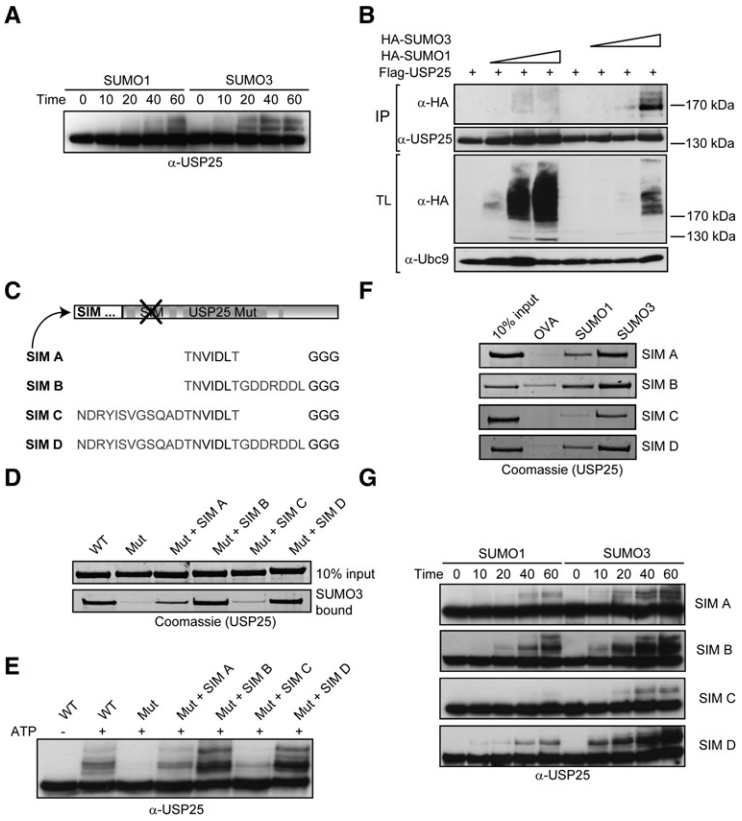

Figure 4. USP25 Is More Efficiently Sumoylated with SUMO2/3

(A) Time course of in vitro sumoylation of USP25 (100nM) with 5 μM SUMO1 or 5 μM SUMO3 in the presence of 10 nM E1 (Aos1/Uba2), 500nM E2 (Ubc9), and 5mM ATP at 30°C.

(B) HEK293T cells were transfected and analyzed as in Figure 3B with increasing amounts of HA-SUMO1 (5, 10, and 20 μg) and HA-SUMO3 (0.25, 0.5, and 1 μg).

(C) Schematic representation of SIM fusions to the N terminus of USP25 (V91A-I92A = Mut) that does not interact with SUMO. Lysine 99 was mutated to arginine to prevent sumoylation of this residue within the transplanted fragment.

(D) Recombinant USP25 SIM fusions (10 μg each) were bound to SUMO3 beads and stained by Coomassie.

(E) In vitro sumoylation assay of SIM fusions as performed in Figure 4A using 5 μM SUMO3.

(F) Binding assay comparing the binding efficiency of USP25 to SUMO1 and SUMO3 as in Figure 1D.

(G) In vitro sumoylation assay of different USP25 SIM fusions as in Figure 4A.

(see Figure S3). As modification in vivo usually involves E3 ligases, we next tested whether SUMO-SIM interaction also contributes to PIASxα-dependent USP25 sumoylation. As shown in Figure 3E, where WT USP25 and SIM mutants were compared for conjugation efficiency, this is indeed the case.

Importantly, the in vitro results can be recapitulated in vivo upon transfection of Flag-USP25 mutants and HA-SUMO3 in HEK293T cells (Figure 3F). While WT USP25 is efficiently conjugated with SUMO3, the SIM mutants show impaired sumoylation that correlates to their noncovalent SUMO-binding properties. Taken together, these results demonstrate that efficient USP25 sumoylation requires the SUMO-SIM interface both in vivo and in vitro.

USP25 Is Conjugated More Efficiently with SUMO2/3

As described above, USP25 requires noncovalent SUMO interaction for covalent conjugation. Since it interacts more efficiently with SUMO2/3 compared to SUMO1 in binding assays, we wondered whether sumoylation of USP25 would also be more efficient with SUMO2/3. To test this hypothesis, we first performed an in vitro time-dependent sumoylation assay in the presence of only E1 and E2 enzymes. As no other known targets have shown paralog specificity in the absence of E3 ligases, we were quite surprised to find that conjugation of USP25 with SUMO3 was about 2- to 3-fold faster than that with SUMO1 (Figure 4A). Preferential SUMO3 conjugation can also be observed when SUMO1 and SUMO3 are present in the same reaction (Figure S4, showing sumoylation of USP25 N-terminal fragment). To control for our reagents, we examined Ubc9 thioester formation and target modification (RanGAP1 and SP100) under the same experimental conditions as in Figure 4A. As expected, neither assay revealed any differences between SUMO1 and SUMO3 (Figures S5 and S6 and data not shown). We next tested whether USP25 would also be a SUMO2/3-specific target in vivo. To be able to compare signal intensities, we transfected HEK293T cells with increasing levels of HA-tagged SUMO1 and SUMO3. After denaturing lysis followed by immunoprecipitation of USP25, we detected the levels of SUMO conjugates with anti-HA antibodies. Even though the signal of HA-SUMO1 conjugated to targets in the total lysates is higher than that of HA-SUMO3, USP25 is better conjugated with SUMO3 in vivo (Figure 4B). Taken together, our findings demonstrate that SIM-mediated sumoylation can serve as a mechanism for paralog-specific sumoylation both in vitro and in vivo.

The SIM Is a Transposable Element for SUMO Binding and Conjugation

We next wanted to address the question whether the SIM of USP25 can work autonomously or whether it depends on structural context. Therefore, we inactivated the SIM within USP25 (Mut = V91A/I92A) and fused different parts of the SIM containing region to the N terminus (Figure 4C). First, we tested the fusion proteins for noncovalent binding to SUMO3 (Figure 4D): the hydrophobic core (SIM A) restores weak binding that is clearly enhanced by the C-terminal acidic stretch (SIM B). The N-terminal stretch, although fully conserved throughout USP25 homologs, has no profound effect (compare SIM A to SIM C and SIM B to SIM D). As it has been shown previously that SIM containing peptides suffice for SUMO interaction (Song et al., 2004; Hecker et al., 2006), we were not too surprised that the SIM motif could bind SUMO in the context of the fusion protein. More interesting was the question whether the SIM motif would also stimulate sumoylation of USP25 when taken out of structural context. For this, we compared the competence of the USP25 SIM fusions for in vitro sumoylation (Figure 4E). Indeed, N-terminal

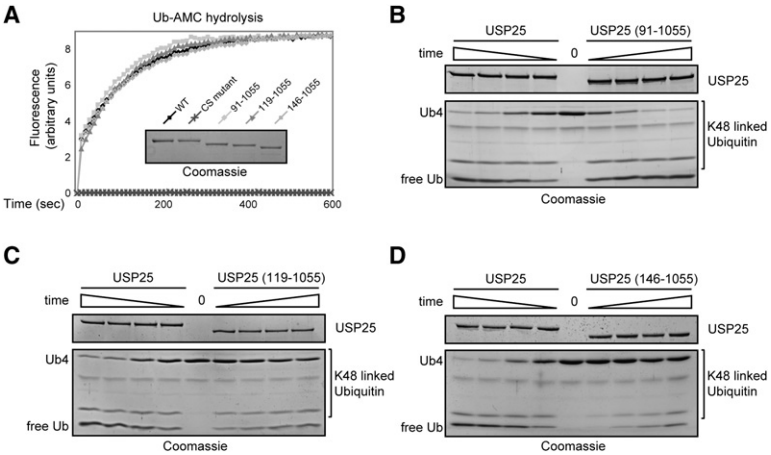

Figure 5. USP25 Requires Its UIMs for Efficient Hydrolysis of Tetra-Ubiquitin

(A) Ubiquitin-AMC was incubated with 200 nM WT or deletion mutants of USP25. Proteins were visualized with Coomassie.

(B) Time course for in vitro deubiquitylation of K48-linked ubiquitin chains (2.5 μM) using 400 nM WT USP25 or USP25 (91–1055), increasing time-points are 10, 20, 40, and 60 min. Proteins were visualized by Coomassie staining.

(C) Time course as in (B) using WT USP25 versus USP25 (119–1055).

(D) Time course as in (B) using WT USP25 versus USP25 (146–1055).

fusion of SIM motifs did restore sumoylation of the SIM-deficient USP25 mutant. As SIM A and SIM C variants were less effective than SIM B and SIM D, efficiency of sumoylation correlated well with efficiency of SUMO binding (compare Figures 4D and 4E).

To gain insights into SUMO paralog discrimination, we then compared each fusion protein for its ability to interact with SUMO1 and SUMO3. Previous studies had pointed to a role of the acidic stretch in paralog discrimination (Hecker et al., 2006). Surprisingly, even the smallest fusion protein (SIM A) showed preferential binding to SUMO3, indicating that the 7 amino acids in SIM A are sufficient for discrimination between SUMO1 and SUMO3 (Figure 4F). Finally, we wanted to strengthen the evidence for a correlation between SUMO binding and SUMO conjugation—as each of the fusion proteins showed preferential binding to SUMO3, it should also prefer SUMO3 for modification. As revealed by the time course shown in Figure 4G, all four fusion proteins are indeed conjugated faster by SUMO3 than by SUMO1.

USP25 Requires Its UIMs for Efficient Ubiquitin Chain Hydrolysis

To better understand the possible role of SUMO binding and conjugation in USP25 function, we initiated a biochemical characterization of this largely unknown protease. First, we tested the efficiency of ubiquitin processing measured by cleavage of ubiquitin-AMC. Full-length untagged USP25 has a K_m of around 5 μM and a k_{cat} of 0.12 s^{-1} (data not shown). The k_{cat} values are nearly identical to those of the catalytic domain of USP2; however, the K_m values are about 10-fold higher than USP2 (Renatus et al., 2006). Therefore, ubiquitin processing, as measured by ubiquitin-AMC cleavage, is most likely not the main catalytic activity in vivo. We next questioned whether the N-terminal UBA domain and UIMs influence the ubiquitin processing activity of USP25. However, based on deletion mutants, none of these domains are required for this activity (Figure 5A). To further characterize USP25, we generated K48- and K63-linked ubiquitin chains with a defined length of 4 ubiquitin moieties and used them for in vitro deubiquitylation assays. Full-length recombinant USP25 is able to efficiently hydrolyze both K48- and K63-tetra ubiquitin (Figure S7 and below). We then asked whether the N-terminal UBA domain and UIMs affect the hydrolysis of K48-linked ubiquitin chains. For this, K48-linked tetra-ubiquitin chains were hydrolyzed in a time-course experiment with either full-length or deletion mutants of USP25. As shown in Figure 5B, the UBA domain of USP25 does not influence the hydrolysis of ubiquitin chains. In striking contrast, deletion of one or two UIM domains impairs ubiquitin chain cleavage. Compare the disappearance of Ub4 chains (and concomitant appearance of free ubiquitin) for WT USP25 and USP25 deletion constructs as shown in Figures 5C and 5D. The deletion mutants display similar behavior toward K63-linked ubiquitin chains (data not shown). In conclusion, these results reveal that the UIMs in USP25 facilitate the hydrolysis of ubiquitin chains.

Sumoylation of USP25 within Its UIM Impairs Ubiquitin Chain Hydrolysis

To address the functional consequences of USP25 sumoylation, we searched for its SUMO acceptor lysines by mutagenesis and subsequent in vitro sumoylation. For better resolution, we used GST-SUMO3 in these experiments. Initial mutagenesis based on the conventional SUMO acceptor site ψKxE (ψ represents a large hydrophobic residue, and x represents any amino acid [Sampson et al., 2001; Lin et al., 2002; Bernier-Villamor et al., 2002]) did not result in the identification of any sumoylation sites. Because USP25 conjugation with SUMO requires the SIM as described above, it seemed likely that sumoylation would take place on lysine residues surrounding the SIM. Upon mutagenesis of conserved lysine residues in proximity to the SIM, we identified lysine 99 and lysine 141 as likely acceptor sites (Figure 6A). Mass spectrometry analysis of a conjugated N-terminal USP25 fragment confirmed lysine 99 as a SUMO acceptor site (Figure S8). Interestingly, lysine 99 and lysine 141 are located within the first UIM and directly adjacent to the second UIM. As shown above, the UIM domains in USP25 are required for efficient hydrolysis of tetra-ubiquitin, but not for ubiquitin-AMC cleavage. We therefore wondered whether sumoylation of USP25 in the UIM domains would have an influence on ubiquitin chain binding and cleavage.

For interaction studies, we expressed and purified a His-tagged N-terminal fragment of USP25 conjugated to SUMO3 (1–146*SUMO3), using a bacterial sumoylation system (Uchimura et al., 2004). The sumoylated fragment was incubated

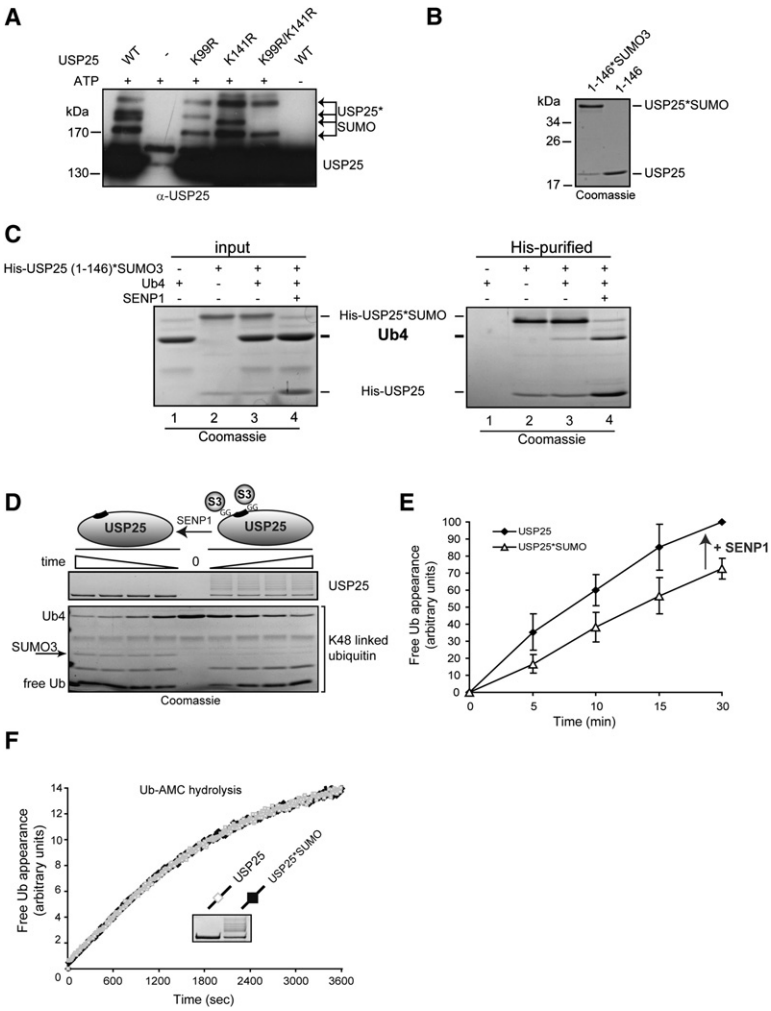

Figure 6. Sumoylation of USP25 Impairs Binding to and Hydrolysis of Tetra-Ubiquitin

(A) USP25 wild-type or mutants (100 nM) were sumoylated in vitro using 10 nM E1 (Aos1/Uba2), 500nM E2 (Ubc9), 2.5 μM GST-SUMO3, and 5 mM ATP for 30 min at 30°C. Samples were analyzed on western blot with anti-USP25 antibodies.

(B) The USP25 N-terminal fragment (1–146) was sumoylated in bacteria and purified. Sumoylated fragment (1–146*SUMO3) was loaded next to the unmodified N-terminal fragment (1–146).

(C) 1–146*SUMO3 (5 μg) was incubated with ubiquitin chains (10 μg) in the presence or absence of GST-SENP1. His-USP25 was purified on Nickel Sepharose and the binding of ubiquitin chains was visualized by Coomassie staining.

(D) In vitro deubiquitylation assay: untagged USP25 was sumoylated with His-SUMO3 for 2 hr at 30°C in the presence of PIASXα and purified on Nickel beads followed by gel filtration. Subsequently, the sample was either treated with GST-SENP1 or left untreated and incubated with 2.5 μM K48-linked Ubiquitin chains for 10, 20, 40, and 60 min. Proteins were visualized with Coomassie.

(E) Quantitation, using Scion Image software, of the free ubiquitin appearing from three independent experiments as performed in Figure S10. Data are represented as average ± standard deviation.

(F) The same batch of protein used in (D) was tested for its activity toward ubiquitin-AMC, and proteins were visualized by Coomassie.

with K48 ubiquitin chains in the presence or absence of an active SUMO isopeptidase fragment (GST-SENP1). Upon enrichment of USP25 fragment on Ni^{2+}-sepharose, samples were analyzed by SDS-PAGE. As revealed in Figure 6C, removal of the SUMO moiety from USP25 increases its interaction with tetra-ubiquitin chains (compare lanes 3 and 4, left and right panel).

To address the question whether sumoylation influences USP25 activity, we modified USP25 with His-SUMO3 in vitro and enriched the modified species by affinity chromatography followed by gel filtration. We were not able to enrich more than 50% USP25*SUMO3 compared to the total pool of USP25 in the sample, which is most likely due to USP25's ability to homodimerize (Figure S9).

To measure activity, we compared sumoylated USP25 with USP25 that was released from USP25*SUMO3 by isopeptidase treatment (GST-SENP1). This procedure allowed us to neglect inactivation that may have occurred during sumoylation and subsequent purification steps. Of note, GST-SENP1 is inactive in deubiquitylation reactions (e.g., compare lanes 3 and 4, left and right panel in Figure 6C for Ub_4). As shown in Figure 6D, sumoylated USP25 is less efficient in ubiquitin chain hydrolysis than deconjugated USP25 (compare decrease in tetra-ubiquitin chains). For quantitative analysis, we repeated the reaction with an excess of ubiquitin chains (Figure S10) and measured the appearance of free ubiquitin. Figure 6E summarizes data from three independent experiments. The reaction rate of sumoylated USP25 is only 67% compared to the desumoylated enzyme (based on the 10 min time point). Given that only 50% of USP25 was sumoylated (based on densitometry), this suggests a residual activity of sumoylated USP25 of 34%. An important control is the comparison of sumoylated and deconjugated USP25 on ubiquitin-AMC. As shown in Figure 6F, their activity is indistinguishable on this substrate. Together with the data presented in Figure 5, our findings suggest that sumoylation of UIM domains inactivates USP25 by inhibiting binding to ubiquitin chains (see Figure 7 for a model). Of note, residual activity of sumoylated USP25 is consistent with the finding that deletion of UIM domains strongly impaired, but did not fully prevent, ubiquitin chain hydrolysis.

DISCUSSION

Paralog-Specific Sumoylation

Simple eukaryotic organisms such as yeast, flies, and worms have a single SUMO protein, while other organisms, including plants and vertebrates, express several distinct SUMO family members (Geiss-Friedlander and Melchior, 2007). While specific functions of individual members are not fully understood, increasing evidence suggests that they have nonoverlapping functions. To name just two examples, SUMO1 haploinsufficiency leads to cleft lip and palate even though SUMO2/3 is much

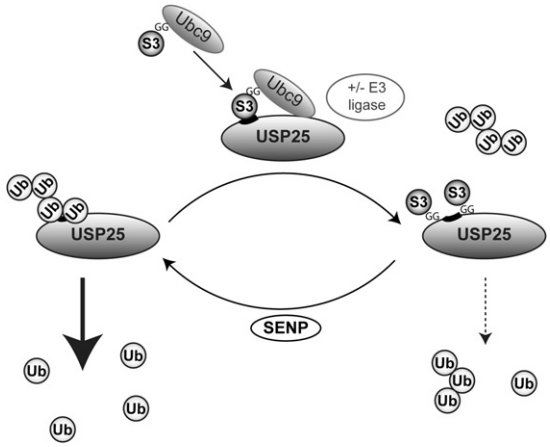

Figure 7. Model for Regulation of USP25 by Paralog-Specific Sumoylation

USP25 is a deubiquitylating enzyme that recognizes its substrate with assistance of the UIMs, upon which ubiquitin chains are hydrolyzed. Sumoylation of USP25 requires its SIM more efficiently interacts with SUMO2/3 (compared to SUMO1) and consequently results in conjugation with preferentially SUMO2/3 paralogs. SIM-dependent sumoylation of USP25 occurs both in the presence or absence of E3 ligases. Sumoylation of USP25 within its UIMs prevents interaction with its substrate and thereby impairs USP25 activity.

more abundant than SUMO1, and SUMO2/3, but not SUMO1, conjugation is increased upon heat shock (Saitoh and Hinchey, 2000; Alkuraya et al., 2006). Moreover, many SUMO targets are preferentially or exclusively modified with specific SUMO paralogs (Vertegaal et al., 2006). Examples are the SUMO1-specific protein RanGAP1 and the SUMO2/3-specific protein Sp100 (Matunis et al., 1996; Mahajan et al., 1997; Saitoh and Hinchey, 2000; Vertegaal et al., 2006). However, reconstitution of target sumoylation in vitro often fails to recapitulate the in vivo findings. This lack of specificity is most pronounced in assays that use only E1 and E2 enzymes (see Figures S5 and S6).

Here we describe a mechanism that contributes to paralog-specific sumoylation of USP25 both in vitro and in vivo: a noncovalent SUMO interaction motif (SIM) in USP25 recruits SUMO2/3 more efficiently, which in turn results in preferential SUMO2/3 conjugation. The underlying mechanism is likely a recruitment of thioester-charged Ubc9 to the target via SUMO/SIM interaction. While at first glance this resembles the recently reported E3 ligase-independent ubiquitylation (Hoeller et al., 2007), we would like to stress that the mechanism described here cooperates with E3 ligase function (compare Figures 3C and 3E). While our work provides clear evidence for a SIM-dependent paralog-specific sumoylation mechanism, a requirement for SUMO-SIM interaction in conjugation is observed in an increasing number of target proteins (Minty et al., 2000; Takahashi et al., 2005; Lin et al., 2006; Kerscher, 2007). We therefore consider it likely that SIMs may contribute to paralog-specific sumoylation for other targets as well. We would, however, like to stress that this mechanism can not be the only one that contributes to paralog-specific sumoylation, as proteins such as RanGAP1 do not seem to bind SUMO noncovalently.

SUMO Paralog-Specific Binding

As described above, the SUMO interaction motif in USP25 belongs to SIM/SBM motifs, which are the only class of SUMO interaction motifs presently known. Previous work on the interaction between SIM containing peptides and SUMO paralogs has already suggested that SIM motifs may work as independent elements in SUMO binding (Song et al., 2004; Hecker et al., 2006). We expanded this idea by demonstrating that the USP25 SIM functions in noncovalent SUMO binding both at its native position and at the N terminus of USP25. Most importantly, we could demonstrate that the USP25 SIM can be taken out of structural context even for SIM-dependent sumoylation. As discussed below, this may have implications for the choice of lysine residues as sumoylation acceptor sites. A second aspect of our work is the characterization of the USP25 SIM in paralog-specific binding. While USP25 binds both to SUMO1 and SUMO2/3, it is more efficient with the latter. Seven amino acids, including the hydrophobic core motif, are sufficient to confer specificity for SUMO2/3. The acidic residues C-terminal of the core motif enhance binding but do not alter paralog specificity. This is quite surprising because previous work suggested that acidic residues flanking the SIM contribute to the discrimination between SUMO1 and SUMO2/3 (Hecker et al., 2006). Future studies, including mutagenesis and NMR-based structural analysis of this and other SIM motifs in noncovalent SUMO binding, will be needed for better definition of the residues that determine paralog-specific binding.

SUMO Acceptor Sites

Conjugation of SUMO to target proteins is usually site specific, such that mutagenesis of the acceptor lysine residue abolishes modification. Such specificity is accomplished by a requirement for specific recognition motifs. Many proteins are modified within the so-called SUMO consensus site, a short motif consisting of the sequence ψKxE (ψ represents a large hydrophobic residue, and x represents any amino acid). Ubc9 recognizes such a motif when presented in extended conformation (Sampson et al., 2001; Lin et al., 2002; Bernier-Villamor et al., 2002). However, an increasing number of proteins turned out to be sumoylated on nonconsensus sites. Examples are PCNA, E2-25K, and Daxx (Hoege et al., 2002; Pichler et al., 2005; Lin et al., 2006). Mutation of nonconventional sites in PCNA and E2-25K abolishes sumoylation, which may argue that Ubc9 does specifically recognize those acceptor sites. However, the SUMO acceptor site in Daxx seems to alter if the preferred lysine is mutated (Lin et al., 2006). It thus appears that specific recognition by Ubc9 may not be the only mechanism that contributes to site selection in sumoylation. Our finding that USP25 is sumoylated on nonconsensus sites that are in close proximity to its SIM motif suggests a second mechanism: when the interaction between thioester-charged Ubc9 and the target is stabilized by a SUMO-SIM interaction (and by target/E3 ligase interactions), Ubc9 may be more flexible to reach nonconsensus sites.

A Role for UIMs in USP25-Mediated Ubiquitin Chain Hydrolysis

Ubiquitin-binding domains can be found in many different proteins and have been implicated in cellular pathways such as

endocytosis, vesicle trafficking, and proteasomal degradation (Hicke et al., 2005; Hurley et al., 2006). Among the proteins that contain ubiquitin-binding domains are also a number of ubiquitin proteases; however, their contribution to enzyme function has remained poorly understood. Only recently has it become apparent that USP5 and USP15 require their ubiquitin-binding Zinc finger for optimal activity (Hetfeld et al., 2005; Reyes-Turcu et al., 2006). In addition, the UIM domain in DUBA was shown to be required for full activity (Kayagaki et al., 2007). Here we provide the second example for a role of UIM domains in isopeptidase function. Deletion of one or both UIM domains of USP25 does not alter C-terminal processing activity but strongly impairs K48 and K63 ubiquitin chain cleavage. Altogether, these findings point to a role for UIM domains in recognition and possibly positioning of ubiquitin chains to the nearby catalytic domain for efficient hydrolysis.

Regulation of Ubiquitylation by Sumoylation

Our finding that USP25 sumoylation within or close to the UIM domains inactivates USP25 adds another mechanism to a surprisingly short list of regulatory events known for deubiquitinating enzymes (Nijman et al., 2005). Mechanistically, the inhibition of USP25 could be a consequence of (1) sterical hindrance of ubiquitin chain binding due to sumoylation of the UIM domains or (2) induction of a conformational change due to intramolecular SUMO-SIM interaction that buries the UIM domains. A similar mechanism was earlier described for TDG, where sumoylation results in a SIM-dependent conformational change that reduces its affinity to DNA (Baba et al., 2005). Structural studies will be required to gain further insights into the exact mechanism of sumoylation-mediated inhibition of USP25. Another key question for future studies is obviously the role and regulation of USP25 sumoylation in vivo.

Sumoylation of USP25 adds an additional building block to crosstalk mechanisms between the sumoylation and ubiquitylation systems. Other examples include sumoylation of E2-25K, which prevents its interaction with the ubiquitin E1 enzyme (Pichler et al., 2005), and the ubiquitin E3 ligase RNF4 that is targeted to its substrates via SUMO/SIM interaction (Xie et al., 2007; Prudden et al., 2007; Sun et al., 2007; Uzunova et al., 2007). Thus, the initial idea that SUMO does not target proteins directly for degradation persists; however, sumoylation may have a profound effect on the ubiquitylation status of proteins by contributing to the function and regulation of ubiquitin-conjugating enzymes, E3 ligases, and deubiquitinating enzymes.

EXPERIMENTAL PROCEDURES

Plasmids

Full-length human cDNA for USP25 was obtained from the German Resource Center for Genome Research (RZPD). USP25 was either HA- or Flag-tagged and cloned into pcDNA3.1. To obtain GST-USP25, USP25 was cloned into the NcoI and BamHI site of pETM30 (EMBL-Heidelberg, Protein Expression Facility). His-TEV-USP25 was generated into pET11a (Novagen). Deletion constructs of USP25 were generated in pET28a (Novagen) with N-terminal Strep and C-terminal His tag except USP25 (1–146) and USP25 (1–654), which contains an N-terminal His-TEV sequence. USP25 SIM (A–D) constructs were generated in pET28a with N-terminal Strep and C-terminal His tag. Plasmids for bacterial expression of untagged SUMO2 (ΔC11) and SUMO3 (ΔC2) were cloned into pET11a, and His-SUMO1, His-SUMO2, and His-SUMO3 were cloned into pET28a. Site-directed mutagenesis of USP25 and SUMO was performed using the Quick-Change site-directed mutagenesis method (Stratagene). Ubc9 for expression in mammalian cells was cloned into the pQE-Tri-System vector (QIAGEN) with an N-terminal Strep and C-terminal His-tag. Plasmids for bacterial expression of Aos1/Uba2, Ubc9, SUMO1, GST-SP100, RanGAP, RanBP2 fragments, ubiquitin, E2-25K, and GST-SENP1 have been described previously (Pichler et al., 2002; Pichler et al., 2005; Bossis and Melchior, 2006). The following plasmids were kind gifts from colleagues: GST-PIAS-1 (Dr. Guntram Suske); GST-PIAS-3 (Dr. Stefan Müller); GST-PIAS-Xα (Dr. Jorma J. Palvimo); HA-SUMO1, HA-SUMO2, and HA-SUMO3 in pcDNA3.1 (Dr. Ron Hay); Uba1 and Ubc13-Mms2 (Dr. Titia Sixma); ubiquitin mutants K63R, K48C, and D77 (Dr. S. Raasi); UCH-L3 (Huib Ovaa); myc-USP25 (G. Marfany); and bacterial expression plasmid for sumoylation in bacteria (pTE1, E2, and SUMO3) (H. Saitoh). All generated constructs were verified by sequencing, and detailed plasmids maps are available on request.

Cell Culture, Transfection, In Vivo Sumoylation, and Immunoblotting

HeLa and HEK293T cells were maintained in DMEM with 10% fetal bovine serum. HeLa cells were transfected using Fugene 6 (Roche Molecular Diagnostics). HEK293T cells were transfected using the calcium phosphate coprecipitation method. For in vivo sumoylation assays, 48 hr after transfection, cells were washed with PBS (10 mM NEM) and lysed in two pellet volumes RIPA buffer (20 mM NaP (pH 7.4), 150 mM NaCl, 1%Triton, 0.5% Sodium-deoxycholate, and 1% SDS) supplemented with protease inhibitors and 10 mM NEM. Lysates were diluted to RIPA buffer containing 0.1% SDS, sonicated, and centrifuged at 4°C (16,000 g for 15 min). The supernatant was incubated with Flag beads (Sigma-Aldrich) for 2 hr at 4°C. After extensive washing, bound proteins were eluted with 2× SDS sample buffer and separated on SDS-PAGE followed by western blotting.

Protein Purification and Ubiquitin Chains

Protein purification for SUMO1, Aos1/Uba2, Ubc9, RanGAP1, GST-SP100, and RanBP2 fragments have been described previously (Pichler et al., 2002, 2004). Purification of SUMO2, SUMO3, or SUMO3 mutants followed the same procedure as SUMO1. His-TEV-USP25 was transformed into *E. coli.* BL21 (Stratagene) was induced with 0.5 mM IPTG for 4 hr at 30°C and lysed in 50 mM NaP (pH 8.0), 300 mM NaCl, 10 mM Imidazole, 1 mM β-Mercaptoethanol, and protease inhibitors, using an EmulsiFlex C3 (Avestin). The 100,000 g supernatant was bound to Nickel Sepharose (QIAGEN) for 2 hr and eluted with 200 mM Imidazole. Protein-containing fractions were desalted on a PD10 column (GE Healthcare) and incubated with TEV protease overnight at 4°C. After subtractive Nickel Sepharose, Untagged USP25 was further purified using gel filtration (Superdex S200, GE Healthcare) and ion-exchange chromatography (MonoQ, GE Healthcare). Deletion mutants and SIM fusion mutants of USP25 (including WT as control) were purified on Nickel Sepharose, followed by gel-filtration. Sumoylation of USP25 (1–146) in bacteria was induced overnight at 25°C (Uchimura et al., 2004) and subsequently purified over Nickel Sepharose, gel filtration, and MonoQ. GST-PIAS-1,-3,-Xα were purified on glutathione-Sepharose (GE Healthcare), eluted with 20 mM Glutathione, and further cleaned using gel filtration. All of the above-mentioned proteins were stored in TB buffer (20 mM HEPES/KOH pH7.3, 10 mM Potassium acetate, 2 mM Magnesium acetate, 0.5 mM EGTA, and 1 mM DTT supplemented with protease inhibitors). Purification of Uba1, E2-25K, Ubc13-Mms2, UCH-L3, ubiquitin, and ubiquitin mutants were essentially purified as described previously (Larsen et al., 1996; Pickart and Raasi, 2005). Ubiquitin chains were generated as described (Pickart and Raasi, 2005). However, for K48 linked chains, the alkylation reaction was omitted, resulting in a wild-type ubiquitin chain with the "top" ubiquitin being (K48C).

In Vitro Binding Assays

SUMO Sepharose was generated by coupling equal amounts of SUMO or SUMO mutants to CnBr-activated Sepharose (Sigma-Aldrich) in Carbonate buffer (0.2 M pH 8.9) at a concentration of 1 mg protein per ml beads. Remaining coupling sites were blocked by incubation with 100 mM Ethanolamine, and beads were stored in TB buffer with 1 mM sodium azide. Cell extracts were bound in TB buffer (+0.1% Triton X-100) or recombinant USP25 in TB buffer

with 0.05% Tween, and 0.2 mg/ml Ovalbumin (Sigma-Aldrich) was incubated with SUMO Sepharose for 2 hr at 4°C. Beads were washed three times in binding buffer (without Ovalbumin) and eluted with 2× Sample buffer.

In Vitro Sumoylation Assays and Deubiquitylation Reactions

In vitro sumoylation reactions have been described previously (Pichler et al., 2002). Large-scale USP25 sumoylation reactions were performed at 30°C in 1 ml with 0.8 μM untagged USP25, 10 nM Aos1/Uba2, 40 nM Ubc9, 100 nM GST-PIASXα, 5 μM His-SUMO3, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM creatine phosphate (Sigma-Aldrich), 0.6 U/ml inorganic pyrophosphatase (Sigma-Aldrich), 0.6 U/ml creatine phosphokinase (Sigma-Aldrich), and 2.5 mM ATP, followed by affinity purification and gel filtration. GST-SENP1 (10 nM) was incubated for 15 min at 30°C, and subsequently, 2.5 μM ubiquitin chains or ubiquitin-AMC was added. Deubiquitylation of K48- and K63-linked ubiquitin chains with USP25 deletion constructs was performed in 20 μl TB buffer with 0.05% Tween at 37°C. Ub-AMC (Biomol) hydrolysis assays were set up in 384-well plates in TB buffer with 0.05% Tween and analyzed with a fluorometer (Fluoroskan Ascent; Labsystems). Kinetic analysis was performed in duplicate by measuring fluorescence at 30°C with excitation at 380 and emission at 450 nm.

Antibodies

Generation of rabbit USP25 antibodies was performed by initial injection of 200 μg GST-USP25 followed by an injection with 200 μg untagged USP25, essentially as described previously (Mahajan et al., 1997). Rabbit polyclonal anti-GST was kindly provided by Dr. Ludger Hengst. Anti-HA and anti-Flag (M2) rabbit polyclonal antibodies were obtained from Sigma-Aldrich, monoclonal anti-HA11 (covance) and rabbit polyclonal anti-Ubc9 (H-81) antibodies were from Santa-Cruz, and polyclonal affinity purified goat anti-RanGAP1 antibodies are described elsewhere (Pichler et al., 2002). Secondary antibodies were obtained from Jackson Laboratories.

SUPPLEMENTAL DATA

The Supplemental Data include ten figures and Supplemental Experimental Procedures and can be found with this article online at http://www.molecule.org/cgi/content/full/30/5/610/DC1/.

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