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SUMO deconjugation is required for arsenic-triggered ubiquitylation of PML

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Abstract

Acute promyelocytic leukemia is characterized by a chromosomal translocation that produces an oncogenic fusion protein of the retinoic acid receptor α (RAR α) and promyelocytic leukemia protein (PML). Arsenic trioxide chemotherapy of this cancer induces the PML moiety to organize nuclear bodies, where the oncoprotein is degraded. This process requires the participation of two SUMO paralogs (SUMO1 and SUMO2) to promote PML ubiquitylation mediated by the ubiquitin E3 ligase RNF4 and reorganization of PML nuclear bodies. We demonstrated that the ubiquitylation of PML required the SUMO deconjugation machinery, primarily the deconjugating enzyme SENP1, and was suppressed by expression of non-deconjugatable SUMO2. We hypothesized that constitutive SUMO2 conjugation and deconjugation occurred basally, and that arsenic trioxide treatment caused the exchange of SUMO2 for SUMO1 on a fraction of Lys⁶⁵ in PML. Based on data obtained with mutational analysis and quantitative proteomics, we propose that the SUMO switch at Lys⁶⁵ of PML enhanced nuclear body formation, subsequent SUMO2 conjugation to Lys¹⁶⁰, and consequent RNF4-dependent ubiquitylation of PML. Our work

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Supplementary Materials

Fig. S1: Profiling the activity of endogenous SENPs in COS7 cell lysates with HA-SUMO2-vme.

Fig. S2: Active SUMO2 removal is required for SUMO-dependent ubiquitylation of PML after As₂O₃ treatment in HEK293A cells.

Fig. S3: Ubiquitylation of PML is RNF4-dependent under the experimental conditions.

Fig. S4: A non-deconjugatable SUMO2 mutant does not reduce RNF4 abundance.

Fig. S5: SENP1 activity is not affected by a non-deconjugatable SUMO2 trimer.

Fig. S6: SENP1 knockdown does not reduce RNF4 abundance.

Fig. S7: SENP5 activity is not required for SUMO-dependent ubiquitylation triggered by As₂O₃ treatment

Fig. S8: SUMO1 is required for the ubiquitylation of PML-IV after As₂O₃ treatment.

Fig. S9: Purified proteins used for in vitro SUMOylation.

Fig. S10: The SUMO2K11R-RING construct does not mimic the SUMO1-RING construct.

Table S1: shRNA sequences

Table S2: Summary of synthetic isotopic peptides

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provides insights into how the SUMO system achieves selective SUMO paralog modification, and highlights the crucial role of SENPs in defining the specificity of SUMO signaling.

Introduction

Modification of proteins by SUMO (small ubiquitin-like modifier) regulates diverse cellular processes, including transcription, replication, chromosome segregation and DNA repair (1–4). Mammalian cells express three SUMO variants or paralogs (SUMO1, SUMO2 and SUMO3) (5). SUMO2 and SUMO3 are ~95% identical and differ from SUMO1 in their ability to form polymers. Because SUMO2 and SUMO3 cannot be distinguished by antisera, they are often referred to as SUMO2/3. SUMO1 shares ~45% sequence identity with SUMO2 and is exclusively conjugated to targets as a monomer. SUMO is conjugated to lysine residues of protein substrates through an enzymatic process that requires a heterodimeric E1-activating enzyme (composed of SAE1 and SAE2 subunits), an E2-conjugating enzyme (UBC9) and, for some substrates, one of a handful of SUMO E3 ligases (6). SUMOylation can be reversed by members of a family of proteases called SENPs (sentrin or SUMO-specific proteases), the more distantly related DESI-1 and -2 (deSUMOylating isopeptidases 1 and 2), and USPL1 (ubiquitin-specific protease-like 1) (6). Thus the number of deconjugating enzymes approximately balances the number of conjugating enzymes. It is generally assumed that deconjugating enzymes exist to recycle SUMO or activate proSUMO.

A current view on the regulation of protein SUMOylation suggests that conjugation occurs through a finely tuned ligation machinery to allow SUMO modification of the right substrate, at the right time. However, this concept does not take into account that conjugation is balanced by deconjugation (7, 8) – in principle a highly dynamic system. We hypothesized that SENPs, acting as editing enzymes in competition with the conjugation machinery, are required for specific SUMO modification of substrates when a stimulus is given. Thus the specificity of SUMOylation would be a combination of conjugation and deconjugation. A growing number of substrates are modified both by SUMO1 and SUMO2, sometimes even on the same lysine (9, 10). These observations suggest that modification of a single lysine by different SUMO paralogs may trigger distinct signals, and thus SUMO deconjugation would control the specificity of signaling.

To test this hypothesis we analyzed a SUMO-dependent event in which both modification of SUMO1 and SUMO2/3 are required to drive a signal: the SUMO driven ubiquitylation of the promyelocytic leukemia protein (PML) triggered by arsenic trioxide (As₂O₃) exposure. PML nuclear bodies are heterogeneous dynamic structures that mediate the post-translational modification of proteins and trigger specific nuclear events in response to cellular stress (11). As₂O₃ promotes the crosslinking of PML (12) and enhances its conjugation to SUMO1 and SUMO2/3. SUMO modification of PML in turn recruits the SUMO-targeted ubiquitin ligase RNF4 which promotes PML ubiquitylation (13, 14) and morphologic alterations of PML nuclear bodies (13). The critical signal required for RNF4-dependent ubiquitylation of PML in cells exposed to As₂O₃ has been proposed to be the synthesis of SUMO2/3 chains on Lys¹⁶⁰ of PML (13, 14); however, the role of SUMO1

modification and how it contributes to this process is a question that remains unanswered. SUMO1 and SUMO2/3 are required for ubiquitylation of PML after As₂O₃ treatment (14). While investigating the role of SUMO paralogs in PML nuclear body morphology we found that the SUMO deconjugating enzyme SENP1 was critical for the response, and we present data that coordinated SUMO deconjugation and conjugation lead to the generation of the specific signal required for the ubiquitylation of PML in cells exposed to As₂O₃.

Results

As₂O₃ does not inhibit SENP activity

The extent of SUMOylation of a protein is, in essence, a balance between conjugation and deconjugation, and so we initially hypothesized that As₂O₃ could inhibit SENP activity to shift the SUMOylation/deSUMOylation balance towards conjugation of PML. To test this hypothesis we analyzed the activity of endogenous SENPs following As₂O₃ exposure of HEK293A (Figure 1) and Cos7 (Figure S1) cells by employing the activity-based probe SUMO2-vinyl methyl ester (vme). SUMO2-vme irreversibly binds the active site of all SENPs, and the activity of the enzymes can be monitored by evaluating a shift of the band corresponding to the enzyme by Western blotting (15). If As₂O₃ inhibits SENPs we would expect reduced labeling of the enzymes with the SUMO-vme probe after treatment. As controls we lysed in the presence of the cysteine alkylator N-ethyl-maleimide (NEM) which prevents labeling of endogenous SENPs, and labeled after heat shock which decreases the activity of SENP1, but not that of SENP6 (12, 16). We did not observe substantial changes in the extent of labeling or mobility shifts in a selection of SENPs (SENP1, SENP6 or SENP5) (Figure 1 and Figure S1, HA-blot), and we concluded that As₂O₃ did not inhibit SENP activity.

Interfering with the dynamic balance of SUMO2 conjugation/deconjugation affects As₂O₃-triggered PML ubiquitylation

Because the enhanced SUMO1 and SUMO2 conjugation observed after As₂O₃ treatment cannot be rationalized by inhibition of SENPs, we utilized specifically engineered SUMO mutants that can distinguish between effects on conjugation and deconjugation to dissect the contribution of each SUMO paralog to As₂O₃-induced ubiquitylation of PML. In these mutants a Pro replaces the natural Gln three residues upstream of the attachment site of SUMO 1 or 2 to their targets, which decreases deconjugating activity 10,000-fold. These forms of SUMO can be conjugated but essentially cannot be deconjugated (7). We utilized these mutants to investigate the role of SUMO dynamics using the most widely studied PML isoform, PML-IV (11).

Because SUMO2 chain synthesis on PML drives its ubiquitylation and nuclear body disassembly (13, 14), we initially anticipated that non-deconjugatable SUMO mutants would enhance the kinetics of PML ubiquitylation. We assessed the effect of the SUMO mutants on the ubiquitylation of PML after As₂O₃ exposure by coexpressing a FLAG-PML-IV construct together with HA-SUMO2-wt or non-deconjugatable HA-SUMO2-Q90P, followed by FLAG immunoprecipitation in CHO-K1 cells, a cell line with low amounts of endogenous PML (17) (Figure 2A and B), and in HEK293A cells (Figure S2A).

Overexpression of non-deconjugatable SUMO mutants did not alter PML protein abundance (Figure 2A and S2A). As expected in cells expressing the non-deconjugatable SUMO2-Q90P, global SUMO2 conjugation (all substrates including PML) was enhanced (Fig. 2A and S2A, lower HA blot), and the basal amount of SUMO2-modified PML was increased, suggesting that the non-deconjugatable SUMO mutant was efficiently conjugated to PML. In contrast, specific SUMOylation of PML in response to As₂O₃ was reduced (Figure 2A and S2A, upper HA-blot). Moreover, ubiquitylation of PML in the presence of SUMO2-Q90P and in response to As₂O₃ was reduced in parallel to a decrease in SUMO1 conjugation (Figures 2A and B and S2A).

These findings seemed counter-intuitive because SUMO2 chain formation has been reported to promote RNF4-dependent ubiquitylation of PML (13, 14), and the SUMO2-Q90P mutant would be expected to enhance this process. To exclude the possibility that our results may involve a different mechanism of ubiquitylation we utilized a dominant negative RNF4 mutant incapable of catalyzing ubiquitylation. Overexpression of this RNF4 mutant in a cell line stably expressing FLAG-PML-IV completely abrogated the ubiquitylation of PML (Figure S3), confirming that the mechanism is RNF4-dependent. Synthesis of SUMO2 chains can trigger activation and auto-ubiquitylation of RNF4 (18). It was therefore reasonable to assume that the decreased ubiquitylation of PML after HA-SUMO2-Q90P overexpression and arsenic exposure was due to decreased RNF4 abundance. To test this possibility we generated a cell line stably expressing GFP-RNF4 (Figure S4A), and monitored protein abundance after overexpression of the different SUMO2 mutants (Figure S4B). Because RNF4 abundance did not decrease after overexpression of HA-SUMO2-Q90P, we concluded that the decrease of PML ubiquitylation observed after overexpression of the SUMO2 mutant was not due to a decrease of RNF4.

It was more likely that the non-deconjugatable SUMO2 mutant blocked the recruitment of RNF4 and subsequent ubiquitylation. We reasoned that non-deconjugatable SUMO2 chains could sequester SENPs and prevent the recruitment of RNF4 and the ubiquitylation of PML. If HA-SUMO2-Q90P chains could sequester SENPs, the activity of SENPs should decrease in the presence of non-deconjugatable SUMO2 polymers. To test this possibility, we generated trimeric SUMO2 variants using wild-type or non-deconjugatable mutant (Figure S5A) and evaluated their effect on the processing of SUMO2-modified RanGAP1 by recombinant full-length SENP1 (Figure S5B). We performed the deconjugation reaction in the presence of a five-fold molar excess of the SUMO2 trimers over SENP1 and we did not observe any substantial delay of SUMO2-RanGAP1 processing (Figure S5B), suggesting that SUMO2-Q90P polymers do not sequester SENP1 or affect its activity.

We next evaluated the effect on non-deconjugatable SUMO1 on As₂O₃-induced ubiquitylation of PML. In cells coexpressing FLAG-PML-IV with non-deconjugatable HA-SUMO1-Q94P, the addition of SUMO2/3 and ubiquitin chains to FLAG-PML-IV after As₂O₃ treatment was not substantially changed (Figures 2C, 2D and S2B). Together these data demonstrate that SUMO deconjugation from PML (specifically SUMO2) plays an active role in the response to As₂O₃.

Arsenic triggers a SUMO paralogue switch from SUMO2 to SUMO1

We were faced with the apparent dilemma that enhanced SUMO2 polymerization reportedly drives ubiquitylation of PML (13, 14) but its deconjugation was equally important (Figures 2 and S2). Armed with this information in combination with the knowledge that non-deconjugatable SUMO1-Q94P did not interfere with the ubiquitylation of PML (Figures 2C, 2D and S2B), we hypothesized that the addition and removal of SUMO2 to a specific lysine residue, followed by the modification of the same lysine residue of PML by SUMO1 is required for As₂O₃-induced ubiquitylation. Possibly a dynamic balance exists with SENPs constantly removing SUMO2 from a specific lysine residue in PML, thereby controlling the accessibility of SUMO1 to this residue. As₂O₃ provides the stimulus for this paralogue switch; SUMO2 is removed from the “switching lysine” in favor of SUMO1, followed by SUMO2 chain formation on a different lysine and subsequent ubiquitylation. The question then became which SENP controlled the SUMO paralogue switch, and which lysine residues provided the basis for these SUMO-dependent cues.

SENP 1 controls SUMO-dependent ubiquitylation of PML

We demonstrated above that non-deconjugatable SUMO2-Q90P serves as a surrogate for inhibition of SENP activity. To rule out off-target effects of SUMO Q/P mutants we validated the requirement of SUMO2 removal for the As₂O₃ response of PML with a complementary tactic. We adopted a loss of function, shRNA-based approach to identify the SUMO protease that controls SUMO-dependent ubiquitylation of PML-IV. We evaluated the effects of specific shRNAs for SENP1, SENP5, and SENP6 on the ubiquitylation of PML after As₂O₃ exposure. We chose to evaluate SENP1 because it is the most active deSUMOylating enzyme and has been suggested to be required for PML nuclear body reorganization (19). SENP6 was chosen because it has been reported to directly regulate SUMO modifications on PML and to control the length of SUMO2/3 chains (18). Its knockdown increases SUMO2/3 and SUMO1 modification of PML (20). SENP5 was chosen as a negative control.

Transfection of HEK293A cells stably expressing FLAG-PML-IV with a lentiviral vector encoding shRNAs specific for SENP1 resulted in >80% knockdown and a concomitant reduction of As₂O₃-induced ubiquitylation of PML (Figure 3A and S6A). The reduction in ubiquitylation was most readily observed in cells transiently transfected with HA-SUMO2 (Figure 3A) and also seen in cells with endogenous amounts of SUMO2, validated with an additional shRNA targeting SENP1 (Figure S6A). Reduction of PML ubiquitylation after SENP6 knockdown (Figure S7) was also observed, validating a report that knockdown of SENP6 triggers RNF4 activation and self-degradation (18). Although the decrease in As₂O₃-induced ubiquitylation of PML after SENP1 knockdown could be a consequence of the decreased RNF4 abundance as seen for SENP6, we rejected this possibility because RNF4 abundance was not altered after SENP1 knockdown in a cell line stably expressing GFP-RNF4 (Figure S6B).

Moreover, knockdown of SENP1 recapitulated the pattern of SUMO modification observed with overexpression of non-deconjugatable SUMO2-Q90P (Figure 2A and S2A). We observed enhanced basal SUMO2 modification of PML-IV, decreased SUMO2 chain

elongation after As₂O₃ treatment, and reduced SUMO1 conjugation and ubiquitylation (Figure 3A and B). In contrast, knockdown of SENP5 did not reduce As₂O₃-triggered PML ubiquitylation, thus assigning specific SENP isoforms to this mechanism (Figure S7).

In addition to recycling SUMO, SENPs are responsible for activating SUMO precursors by removing their C-terminal tails, and it was formally possible that the reduction in the SUMOylation of PML induced by SENP1 knockdown was due to decreased recycling of SUMO1 and SUMO2. However, this possibility was ruled out because we performed SENP1 knockdown experiments in cells stably expressing PML derivatives, with or without overexpression of mature SUMO that does not require processing (Figure 3A).

As₂O₃ promotes the transfer of PML proteins from the nucleoplasmic fraction to the nuclear matrix to drive PML aggregation and subsequent SUMOylation (12, 21). Because we demonstrated that SENP1 was required to trigger SUMO-dependent ubiquitylation of PML after As₂O₃ treatment, we next reasoned that SENP1 activity may affect the recruitment of PML to the nuclear matrix after As₂O₃ treatment. However, knockdown of SENP1 did not affect the rate of PML recruitment to a detergent insoluble fraction (Fig. 3C). Together with the knockdown data, we reasoned that SENP1 controls the SUMO paralogs switch on PML.

SENP1 controls PML nuclear body formation after As₂O₃ exposure

Our results showed that SENP1 activity was required for the ubiquitylation of PML after As₂O₃ treatment. Because As₂O₃ induces the formation of PML nuclear bodies (21), we next asked if the activity of SENP1 was required for the previously reported changes (13) in PML nuclear body dynamics triggered by As₂O₃. HEK293A cells were transfected with shRNA targeting SENP1, and PML nuclear bodies were monitored through immunofluorescence using an antibody targeting all PML isoforms.

As₂O₃ triggered a rapid increase of the number of PML nuclear bodies that slowly decreased over time (Figure 4A and B), consistent with previous findings (13). The changes in nuclear body dynamics were reflected in the appearance of high molecular weight endogenous PML species (Figure 4C). Knockdown of SENP1 delayed the appearance of high molecular weight PML forms (Figure 4C); however, a fraction of endogenous PML proteins persisted after 24 hours of As₂O₃ treatment in agreement with recent findings (22). Moreover, knockdown of SENP1 led to a significant decrease in the amount of nuclear bodies formed after one hour of As₂O₃ treatment (Figure 4A and B). Our findings indicate that the activity of SENP1 is required for formation and stabilization of PML nuclear bodies after As₂O₃ treatment.

The SUMO2/3 chain-forming lysine in PML is Lys¹⁶⁰ and the SUMO-switching lysine is Lys⁶⁵

PML can be modified by all three SUMO paralogs (23) on three dominant lysine residues (65, 160 and 490) (24), and it has been suggested that a SUMO2/3 chain emanating from Lys¹⁶⁰ is required to recruit the E3 ubiquitin ligase RNF4 which in turn ubiquitylates PML (14). Because we found that active removal of SUMO2 from PML was required to allow SUMO1 modification and RNF4-dependent ubiquitylation, we concluded that one of the PML lysines must be conjugated by SUMO1 to promote SUMO2-dependent ubiquitylation

of PML after As₂O₃ treatment and that SENP1 was the enzyme that controlled the specific SUMO paralog that was attached to this SUMO-switch lysine.

To confirm the requirement of SUMO1 for As₂O₃-triggered ubiquitylation of PML we knocked down SUMO1 with two independent shRNAs in a cell line stably expressing FLAG-PML-IV and monitored the modification of PML after As₂O₃ treatment (Figure S8). SUMO1 knockdown resulted in a strong reduction of SUMO2 conjugation and consequent ubiquitylation of PML after As₂O₃ treatment (Figure S8), confirming the vital role of the SUMO1 paralog in regulating PML ubiquitylation.

To identify the lysine modified by SUMO1 that promotes PML ubiquitylation we generated stable CHO-K1 cell lines carrying different PML lysine→arginine point mutations. Mutation of residue 160 substantially decreased As₂O₃-triggered SUMO2/3 PML modification and subsequent ubiquitylation (Figure 5A). As₂O₃ treatment still triggered SUMO1 modification of this PML mutant, suggesting that other lysines, most likely Lys⁶⁵ and Lys⁴⁹⁰, were modified by SUMO1 after As₂O₃ treatment. Mutation of Lys⁴⁹⁰ did not affect ubiquitylation triggered by As₂O₃ (Figure 5A), suggesting that modification of this residue played a less crucial role in SUMO-dependent ubiquitylation of PML. Mutation of Lys⁶⁵ slightly decreased ubiquitylation and strongly reduced SUMO2/3 and SUMO1 conjugation of PML (Figure 5A). This result implied that Lys⁶⁵ could be the SUMO switching lysine that was dynamically modified by SUMO2/3 under basal conditions with a switch to SUMO1 following As₂O₃ treatment. The strong reduction of SUMO2/3 conjugation observed with this PML mutant prompted us to hypothesize that the switching residue Lys⁶⁵ controls SUMO2/3 addition to the chain forming Lys¹⁶⁰.

As an orthogonal way to assess SUMOylation of specific lysines and to exclude the possibility that the lysine/arginine mutations of PML have unexpected consequences, we utilized quantitative mass spectrometry to assess PML obtained from HEK293A cells stably expressing FLAG-PML-IV after As₂O₃ treatment. To identify SUMO-modified lysines of PML we immuno-precipitated SUMO-modified PML from cells treated or untreated with As₂O₃, and digested the eluted material with chymotrypsin to generate peptides of appropriate size with a short “SUMO tag”. Trypsin was added in a separate double digestion to generate SUMO-modified peptides of the appropriate size around Lys⁴⁹⁰. To quantitate the degree of SUMOylation, we obtained synthetic branched AQUA peptides corresponding to the chymotryptic or tryptic/chymotryptic peptides encompassing Lys⁶⁵, Lys¹⁶⁰, or Lys⁴⁹⁰ modified by SUMO1 or SUMO2/3. Before performing mass spectrometry analysis we spiked known amounts of the AQUA peptides into the PML digested mixture to enable us to quantify the amount of modified lysines in the PML mixture (Figure 5B,C).

We observed equivalent SUMO2/3 modification of Lys⁴⁹⁰ in the untreated and As₂O₃-treated sample (Figure 5C), suggesting that modification of this residue in PML does not play a crucial role in the As₂O₃ response. We noted an increase in SUMO2/3 addition to Lys⁶⁵, and a much more substantial increase at Lys¹⁶⁰ after As₂O₃ treatment (Figure 5B,C). This result agreed with the data obtained with the Lys→Arg mutants and demonstrated that Lys¹⁶⁰ was the major SUMO2/3 modification site of PML triggered by As₂O₃, providing further evidence that Lys¹⁶⁰ is a likely candidate for the chain-forming lysine.

Unfortunately, the low amount of SUMO1 conjugated peptides observable in our experiments precluded us from unambiguously assigning a switching lysine. However, the Lys/Arg PML mutation strategy suggested that Lys⁶⁵ on the RING domain of PML was the switching lysine, the residue that needed to be modified by SUMO1 to drive SUMO2 chain synthesis. We concluded that As₂O₃ enhanced both SUMO2/3 and SUMO1 modification of Lys⁶⁵ in PML; however, only the fraction modified by SUMO1 would be able to drive the ubiquitin signal. To test this we created constructs of PML with SUMO1 or SUMO2 fused to the RING domain, simulating attachment to Lys⁶⁵.

SUMO1 modification of the PML RING domain promotes SUMO2 conjugation to Lys¹⁶⁰

The question arising from our findings was why SUMO1 modification of PML was required for SUMO-dependent ubiquitylation of the protein triggered by As₂O₃ treatment. We hypothesized that SUMO1 modification of Lys⁶⁵ was required to promote SUMO2 modification on Lys¹⁶⁰ and to test this hypothesis we reconstituted the SUMO modification of PML *in vitro*.

We simulated SUMO modification of Lys⁶⁵ by fusing the different SUMO paralogs to the N-terminus of the RING domain of PML (residue 49 - Figures S9 and 6A). With these constructs we tested the requirements for SUMO conjugation to Lys¹⁶⁰. In the presence of the conjugating enzymes, HA-SUMO1-RING-B1-B2 was conjugated to SUMO2, which was selective for Lys¹⁶⁰ (Figure 6B). In contrast, SUMO2 was conjugated to wild-type or K160R HA-SUMO2-RING-B1-B2 constructs at similar extents, demonstrating that SUMO1, not SUMO2, specifically directed the conjugation of SUMO2 to Lys¹⁶⁰ (Figure 6B). Presumably SUMO2 was conjugated to another lysine, possibly the SUMO2 moiety in the K160R construct, most likely at the chain extension site Lys¹¹ (25). Because UBC9 has similar affinities for SUMO1 and SUMO2 (26) we hypothesized that the HA-SUMO2-K11R-RING-B1-B2 construct carrying a mutation on the SUMO2 moiety to prevent SUMO2 chain formation might behave similarly to HA-SUMO1-RING-B1-B2. Indeed, the HA-SUMO2-K11R-RING-B1-B2 was conjugated to SUMO2, regardless of whether there was a Lys or Arg at position 160 (Fig. S10). This result suggested the presence of another SUMO2 modification beyond Lys¹¹ and Lys¹⁶⁰. However, because SUMO2 chain-formation of Lys¹⁶⁰ of PML is critical for RNF4-dependent ubiquitylation, we did not further investigate additional SUMOylation sites. In control experiments RING-B1-B2 without a SUMO moiety fused to the N-terminus could not serve as a SUMOylation substrate (Figure 6B). We concluded that SUMO2 attachment is indeed on Lys¹⁶⁰ and crucially depends on a RING domain modified by SUMO1.

Finally we reconstituted our model in cells. We fused the different SUMO paralogs to the N-terminus of the RING domain of PML-IV, mutated the last glycine residue of SUMO to alanine to prevent removal by SENPs, and generated stable cell lines carrying the different constructs. Removal of the poly-proline rich domain (aa 1-48) from full length PML-IV did not substantially affect As₂O₃-triggered ubiquitylation (Figure 6C). Mutation of Lys⁶⁵ strongly decreased SUMO2 conjugation and ubiquitylation after As₂O₃ treatment, recapitulating the *in vitro* results. Moreover, fusion of SUMO1 at the N-terminus of the RING domain of PML-IV restored ubiquitylation of the Lys⁶⁵ mutant in response to As₂O₃,

whereas fusion of SUMO2 to the RING domain of PML-IV induced a substantially lower amount of ubiquitylation (Figure 6C). Guided by the *in vitro* experiment described above, we generated a cell line stably expressing a chain-defective SUMO2 construct (SUMO2-K11R-PML) to determine whether chain-defective SUMO2 could mimic SUMO1. This mutant was ubiquitylated less efficiently than SUMO1-PML after As₂O₃ treatment (Figure 6D). These data are consistent with a requirement of the conjugation SUMO1, but not SUMO2/3, to Lys⁶⁵ in the RING domain to promote addition of SUMO2 chains on Lys¹⁶⁰ with consequent promotion of the ubiquitin signal.

Discussion

The highly dynamic nature of the SUMO system is not a new concept; however, the concept that deconjugation could control specificity of SUMO paralog modification has not been explored. We postulated that for certain targets specificity may be controlled by deconjugation rather than targeted conjugation (Figure 7A). In this work, we present evidence for a deconjugation controlled SUMO signal. In our working model (Figure 7B), SENP1 would act as an editing enzyme that reverses SUMO2 conjugation to Lys⁶⁵ to allow specific SUMO1 modification when the right stimulus occurs.

The paralog switch model is consistent with the notion that SUMO1 conjugation to the RING domain of PML promotes SUMO2 modification of Lys¹⁶⁰ in PML. In support of this model, UBC9 interacts with the RING domain of PML (27) and with SUMO through a region distant from the active site that does not influence UBC9/SUMO thiol-ester formation (26, 28). We propose that under basal conditions, UBC9 promotes SUMO2 conjugation to Lys⁶⁵ which is constantly counterbalanced by SENP1. Upon As₂O₃ exposure, and in a yet unidentified mechanism, SUMO1 modification of the RING domain of PML occurs (Figure 7B). Because of strategically placed lysine residues in its N-terminal extension, SUMO2 can form chains, but SUMO1 cannot. Because SUMO1 cannot serve as a scaffold for chains, UBC9 defaults to conjugating SUMO2 to Lys¹⁶⁰, the chain forming lysine, which consequently leads to the recruitment of RNF4 for the ubiquitylation of PML. This process is thus an inherent property of the different chain forming abilities of SUMO paralogs linked to the switching Lys⁶⁵. Our findings leave room for an additional interpretation. We were intrigued by the possibility that SUMO1 could behave also as SUMO2/3 chain terminator to prevent SUMO2/3 chains on Lys⁶⁵, thereby favoring SUMO2/3 conjugation to Lys¹⁶⁰ in PML after As₂O₃ treatment. This interpretation would explain why we observed a slight increase in SUMO2/3 modification on Lys⁶⁵ in the mass spectrometry experiments and why the SUMO2-PML construct was still ubiquitylated (although to a much lesser extent) after As₂O₃ treatment. However, the results obtained with the SUMO2 chain defective PML fusion variants both in the *in vitro* and cellular experiments do not support this hypothesis. The SUMO2-K11R construct does not appear to behave like SUMO1 (Figure S10 and 6D).

We considered the possibility that the requirement for SUMO2 conjugation/deconjugation may relate to another protein, and not directly PML, but the Lys→Arg mutagenesis data, primarily the K65R mutant, argues for the direct participation of PML in the SUMO paralog switch. To account for the importance of SUMO2 deconjugation in driving the ubiquitin

signal, we propose that the PML/SUMO/ubiquitin system would be in a balanced equilibrium in the basal state, in which the SUMO2 conjugation machinery is dynamically counteracted by deconjugation through SENP1. This highly dynamic state is not a unique feature of PML but it has been previously proposed to be a common property of the SUMO network (7, 8, 29). Under this condition, the signal cannot propagate because SUMO2/3 chain synthesis on Lys¹⁶⁰ is not favored. Two events are needed to promote the paralog switch on Lys⁶⁵. First, the crosslinking of PML induced by As₂O₃ alters the SUMO conjugation/deconjugation balance. Second, because As₂O₃-induced PML crosslinking may protect the substrate from SUMO deconjugation, SUMO1 modification of PML Lys⁶⁵ would now promote the synthesis of SUMO2/3 chains on Lys¹⁶⁰ (Figure 7B). This model is consistent with our data showing propagation of the ubiquitylation signal by the non-deconjugatable SUMO1-Q94P. The modification by SUMO1 is essential to provide the cues to synthesize SUMO2/3 chains on Lys¹⁶⁰ and promote the signal. However, at the same time, we speculate that PML crosslinking coupled with SUMO1 conjugation may also be required to stabilize the PML nuclear bodies, preventing prompt SUMO deconjugation and premature disassembly. Future studies will be needed to demonstrate the exact nature of the response of PML to As₂O₃ and these could include overcoming the technical challenge of purifying intact PML nuclear bodies and exploring the role of different PML splicing variants in propagating the SUMO-dependent ubiquitin signal.

The reversible modification of Lys⁶⁵ leading to modification of Lys¹⁶⁰ seems like a complex way to organize cell signaling, and searching for an explanation we are struck by the possibility that SUMO modification of PML follows a stochastic process. In this scenario, a constant conjugation/deconjugation (futile) cycle of SUMO2 on Lys⁶⁵ of PML dominates unstimulated conditions, but upon As₂O₃ treatment, which leads to PML oxidation and clustering (12), sufficient SUMO1 is now conjugated to drive the signal. We postulate that it is the altered conformation of PML, not a change in the ligation machinery, which permits SUMO1 ligation onto Lys⁶⁵ and subsequent SUMO2/3 chain formation at Lys¹⁶⁰. Thus, the substrate PML determines when it is time to be modified by the initiating signal, SUMO1. This hypothesis has strong similarity to the proposed mechanisms of a calcium/calmodulin-dependent protein kinase II stochastic switch implicated in long-term memory in the nervous system (31), and of molecular noise in stochastic bacterial gene activation networks (32). We do not know whether this process, explaining the SUMO paralog switch of PML by stochastic means, extends to other proteins, but the hypothesis seems at least as harmonious as the concept that the many hundreds of proteins conjugated and deconjugated by SUMO are all specifically regulated by so few ligases.

Materials and Methods

Plasmid Constructs

PML-IV coding sequence, accession number AF230406, was amplified from a human keratinocyte cDNA library. The PCR product was cloned in pcDNA3 (Life Technologies) and pEGFP-N2 (Clontech) with an N-terminal FLAG tag. Lysine to arginine mutants of PML-IV and ubiquitin were generated with site-directed mutagenesis using primers carrying the specific mutation. RanGAP1 N in pET28b, proSUMO2 in pET28, SUMO1, SUMO2

and SUMO2-Q90P constructs cloned into pcDNA3 with an N-terminal HA-tag were previously described (7, 33). SUMO1-Q94P was generated using a reverse primer carrying the specific mutation. Full length SENP1 construct (7) was subcloned in pGEX4T1. pTE1E2S2 was kindly provided by Dr. Hisato Saitoh, Department of Biological Sciences, Kumamoto University (34). pTE1E2S2-Q90P was generated with site-directed mutagenesis using a primer carrying the specific mutation. pTYB2-HA-SUMO2 was kindly provided by Dr. Keith Wilkinson, Department of Biochemistry, Emory University, Atlanta, GA (35). Mouse RNF4 and RNF4-C177S constructs cloned in pEGFP-C1 were kindly provided by Dr. Tony Hunter, Salk Institute for Biological Studies, La Jolla, CA (36). PML RING-B1-B2 (aa 49-236) and SUMO-RING-B1-B2 (SUMO1 aa 1-97 + PML aa 49-236 or SUMO2 aa 1-93 + PML aa 49-236) constructs were cloned into the pET29b expression vector (Novagen) with an N-terminal HA-tag introduced with the forward primer. UBC9 and SUMO E1 (Aos1/Uba2) expression constructs were kindly provided by Dr. Frauke Melchior, Universität Heidelberg, Heidelberg, Germany (37). FLAG-SUMO-PML-IV fusion mutants were generated with overlapping PCR fusing SUMO1 (aa 1-97) or SUMO2 (aa 1-93) with PML-IV (aa 49-633). FLAG tag was introduced at the N-terminus of each construct with the forward primer and last glycine of each SUMO paralog was mutated to alanine to prevent SUMO removal. The entire list of primers is available upon request. All constructs described were verified by DNA sequencing.

Cell Culture, Stable Cell lines and Treatment

HEK293A and Cos7 cells were 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. CHO-K1 cells were maintained in HAM's F-12 medium (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Stable cell lines expressing GFP-RNF4, FLAG-PML-IV-GFP wt and mutants, FLAG-PML-IV wt and mutants, FLAG-SUMO-PML-IV fusion mutants cloned respectively in pEGFP-C1, pEGFP-N2 and pcDNA3 were obtained by transfecting HEK293A or CHO-K1 with NanoJuice Transfection Reagent (Novagen) according to the manufacturer's instructions. Transfected cells were selected for two weeks with 500 µg/ml G418 and propagated after selection. As₂O₃ (Sigma) was dissolved in 1M NaOH, diluted and pH adjusted to get a stock concentration of 5 mM, pH 8. As₂O₃ was used at the final concentration of 2 µM in all the experiments.

RNA Interference

Three shRNAs per gene were designed and cloned in pLKO.1 (Sigma). The clones that showed the strongest knockdown were used for the experiments. The sequences for the shRNAs can be found in table S1. Cells were transfected using the corresponding shRNA with NanoJuice Transfection Reagent (Novagen) according to the manufacturer's instructions, selected for 72 h with 1 µg/ml puromycin and treated accordingly.

Immunoblotting and Immunoprecipitation

Treated cells were scraped, washed twice in ice cold PBS, and pellets were frozen at -80°C until all the time points were collected. Cell pellets were resuspended in RIPA buffer (50

mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% TX100, 0.5% SDS), supplemented with 50 mM N-ethyl-maleimide (NEM, Pierce) and protease inhibitors (transepoxy succinyl-L-leucylamido(4-guanidino) butane, 3,4-dichloroisocoumarin, leupeptin and MG-132, all 10 μ M). After resuspension, lysates were boiled for five minutes to extract PML proteins from the nuclear matrix and disrupt protein complexes, diluted 1:3 in TBS with 1% TX100, and passed through a 27G needle to shear the DNA. Lysates were cleared by centrifugation and the supernatants were quantified through BCA protein assay (Pierce) and normalized before performing immunoblotting and immunoprecipitation. FLAG immunoprecipitation was performed by incubating cell lysates overnight at 4°C with anti-FLAG agarose beads (M2, Sigma), followed by washing three times in ice cold TBS + 1% TX100. Beads or total lysates were then mixed with SDS loading buffer, boiled and subjected to Laemmli SDS-PAGE (8% or 12% gels), followed by transfer to a nitrocellulose membrane for Western blotting. Primary antibodies were detected by fluorophore-coupled secondary antibodies (LiCOR), and signal was quantified using the Odyssey system. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Immunofluorescence

Cells were grown on coverslips, treated, washed twice with PBS, and fixed for 20 min in 4% paraformaldehyde, 2% sucrose in PBS at room temperature. Permeabilization and blocking was performed at room temperature for 30 min in PBS, 0.4% TX100, 2% BSA. Staining with primary antibodies was performed for 1 h at room temperature or overnight at 4°C in PBS containing 0.1% TX100 and 0.5% BSA. Coverslips were washed multiple times in PBS, 0.2% TX100, 1% BSA and stained with secondary antibodies conjugated with AlexaFluor 568 (Life Technologies). Coverslips were mounted for imaging with Vectashield containing DAPI. Images were acquired using a LSM 710 NLO Zeiss Multiphoton Laser Point Scanning Confocal Microscope. Images shown represent single Z-slice projections. Counting of nuclear bodies was performed in a blinded setup. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Mass-spectrometry analysis of the SUMOylation status of Lys⁶⁵, Lys¹⁶⁰ and Lys⁴⁹⁰ in PML

HEK293A cells stably expressing FLAG-PML-IV were grown and treated with 2 μ M As₂O₃. Cells lysis and FLAG immunoprecipitation were performed using the protocol described in the previous section but without NEM and in the presence of 50 mM iodoacetamide (IAA). PML was eluted from the beads using 5 mg/ml of 3XFLAG peptide and incubation on ice. Eluted samples were subjected to Laemmli SDS-PAGE and PML material excised from the gel as single slice. Gel pieces were diced into 1 mm cubes and destained in 50 mM ammonium bicarbonate (AmBic) in 50% acetonitrile (ACN) (v/v) for 15 min or until clear. Gel pieces were dehydrated with 30 μ L of 100% ACN for 5 min, the liquid was removed, and the gel pieces were rehydrated in 5 mM DTT and incubated at 50°C for 60 min. Gel pieces were again dehydrated in 100% ACN, liquid was removed and gel pieces were rehydrated with 12.5 mM IAA. Samples were incubated at room temperature in the dark for 20 min. Gel pieces were washed with 50 mM AmBic and dehydrated with 100% ACN. Gel pieces were rehydrated with 50 μ M sequencing grade chymotrypsin (Pierce Biotechnology, Rockford, IL), resuspended in 50 mM Ambic on ice

for 1 h. Excess liquid was removed and gel pieces were digested with chymotrypsin at 37°C overnight. Peptides were extracted with 50% ACN/0.1% formic acid, followed by 100% ACN/0.1% formic acid. Peptides were dried to completion and resuspended in 2% ACN/0.1% formic acid for the chymotrypsin alone samples or resuspended in 100 µl 50 mM AmBic containing trypsin for 4 h at 37°C. Samples were dried down and combined with custom isotopically labeled synthetic PML peptides representing versions of chymotryptic SUMOylated Lys⁶⁵, Lys¹⁶⁰, or chymotryptic + tryptic SUMOylated Lys⁴⁹⁰. Arginine or lysine carried the isotopic label adding 10.0083 Da or 8.0142 Da to the mass of the peptide, respectively. This is indicated by lowercase r* or k* and the SUMO tag modified Lys is indicated in bold. The synthetic peptides were reduced and cysteines were alkylated with IAA (+57.021464) prior to spiking them into the PML peptide extract. A summary describing the synthetic peptides is found in Table S2.

Samples were injected in duplicate through an auto-sampler onto a nanoAcquity UPLC (Waters, Milford, MA) over a 2 h gradient and analyzed on-line through nanospray ionization into an LTQ Orbitrap-Elite mass spectrometer operated in data-dependent mode. Full MS scans were analyzed in the Orbitrap at 60,000 resolution and the top 15 most abundant ions were selected for fragmentation in the ion trap. Synthetic peptides were each added at a concentration of 250 fmol per injection. Areas were integrated for the isotopic SUMOylated peptides in Xcalibur and compared to their isotopically light counterpart to obtain quantification of SUMOylation present at these sites.

Protein purification and *in vitro* SUMOylation

All recombinant proteins were expressed in E. coli BL21 (DE3) (Novagen). Protein expression was induced at OD₆₀₀ = 0.6 with 0.2 mM IPTG for 5 h at 25°C. PML constructs (aa 49-236) were expressed in the presence of 100 µM ZnCl₂. To generate SUMO2-modified His-RanGAP1 N we coexpressed the RanGAP1 construct with pTE1E2S2. Tri-SUMO2-wt and Q90P were generated coexpressing proSUMO2-His tagged with pTE1E2S2 or pTE1E2S2-Q90P. His-tagged proteins were purified using Chelating Sepharose Fast Flow (GE) charged with 100 mM NiSO₄, and eluted with a 20–200 mM gradient of imidazole in 50 mM Tris pH 8, 100 mM NaCl. Tri-SUMO2-wt and Q90P were subsequently purified by anion exchange chromatography on a Mono Q column (GE). GST-tagged full length SENP1 was purified using Glutathione Sepharose 4B (GE) and eluted with 50 mM reduced glutathione in 50 mM Tris pH 8, 100 mM NaCl. Recombinant UBC9 and SUMO E1 (AOS1/UBA2) were purified using a procedure described in the literature (37). HA-SUMO2-vme was prepared using a procedure described in the literature (35). Protein purity was evaluated through SDS-PAGE and concentration was determined through BCA Protein Assay (Pierce). *In vitro* SUMOylation was performed in 20 µl reactions containing 2 µM substrate, 5 µM SUMO2, 300 nM AOS1/UBA2, 1 µM UBC9, 1X Energy Regeneration Solution (R&D) in 50 mM Tris pH 7.5. Reactions were incubated at 37°C for the corresponding time and analyzed by immunoblotting.

Antibodies

Anti-PML for Western blotting and immunofluorescence (PG-M3, Santa Cruz), anti-FLAG (M2, Sigma), anti-β-tubulin (ab6046, Abcam), anti-GAPDH (#2118, Cell Signaling), anti-

GFP (D5.1 XP, Cell Signaling), anti-HA for Western blotting (clone 16B12, Covance), anti-ubiquitin (clone FK2, Enzo LifeSciences, and whole antiserum U5379, Sigma), anti-SUMO1 and anti-SUMO2 antisera were prepared in house using mature recombinant SUMO1 and SUMO2 as antigen (7). Anti-SENP1 and anti-SENP6 were prepared in house using residues 419–643 as antigen for SENP1 and residues 628–1112 for SENP6, followed by affinity purification. SENP5 antiserum was kindly provided by Dr. Heidi McBride, McGill University Montreal Canada (38).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Profiling the activity of endogenous SENPs in HEK293A cells lysates with HA-SUMO2-vme

HEK293A cells were treated with As_2O_3 or heat shocked. Whole cell lysates prepared with or without the HA-SUMO2-vme activity-based probe were separated with SDS page, transferred to nitrocellulose and blotted with the antibodies indicated. The data is representative of two independent experiments.

Figure 2. Active removal of SUMO2 is required for SUMO-dependent ubiquitylation of PML after As₂O₃ treatment

A) Western blot analysis of FLAG immunoprecipitates from CHO-K1 cells cotransfected with FLAG-PML-IV and HA-SUMO2-wt or Q90P. The data is representative of three independent experiments. B) Quantification of the relative ubiquitylation of FLAG-PML-IV from the data in (A). C) Western blot analysis of FLAG immunoprecipitates from CHO-K1 cells cotransfected with FLAG-PML-IV and HA-SUMO1-wt or Q94P. The data is representative of three independent experiments. D) Quantification of relative ubiquitylation

of FLAG-PML-IV from the data in (C). Relative ubiquitylation values represent the average of at least three independent experiments $\pm$ SEM. $P < 0.05$ Student's two-tailed t test.

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Figure 3. SENP1 activity is required for PML-IV ubiquitylation after As₂O₃ treatment
 A) HEK293A cells stably expressing FLAG-PML-IV-GFP were cotransfected with an shRNA for SENP1 or empty pLKO vector and HA-SUMO2, selected with puromycin and treated with As₂O₃. FLAG immunoprecipitates were immunoblotted with the antibodies indicated. The data is representative of at least three independent experiments. B) Relative ubiquitylation values were obtained after densitometry quantification of the bands and calculation of the ratio of the ubiquitin signal over the loading control. Ubiquitylation of the control sample when empty pLKO vector was transfected was set as 1. Each value

represents the average of at least three independent experiments $\pm$ SEM. $P < 0.05$ Student's two-tailed t test. C) HEK293A cells stably expressing FLAG-PML-IV-GFP were transfected with a shRNA for SENP1 or empty pLKO, selected with puromycin and treated with As_2O_3 . The soluble fraction was separated from insoluble by centrifugation and PML amounts analyzed by Western blot. The data are representative of two independent experiments

Figure 4. SENP1 activity is required for PML nuclear body formation after As₂O₃ treatment
A) HEK293A transfected with an shRNA for SENP1 or empty pLKO vector were selected with puromycin, plated on coverslips and treated with As₂O₃. Changes in PML nuclear body formation were assessed through immunofluorescence using an antibody specific for PML. Scale bar = 10 μm. B) Statistical analysis of the effect of SENP1 knockdown on PML nuclear body formation. Values show the average number of PML nuclear bodies per cell ± SEM for each sample (n = 100 cells per sample; two independent experiments.). Student's t test showed that the effect of SENP1 knockdown was statistically significant 1 h after As₂O₃ stimulation (p < 0.0001). C) Western blot analysis of whole cell lysates obtained from the samples used for immunofluorescence with the antibodies indicated. The data is representative of three independent experiments.

B

	Peptide Sequence	Untreated (fmol)	As ₂ O ₃ (fmol)	Difference (fmol)
K65-SUMO2	RCQQCQAEAKCPKLLPCLHTL (QQQTGG)	0	0.2	0.2
K160-SUMO2	FLKHEARPL (QQQTGG)	20	34.6	14.6
K490-SUMO2	KVIMKESEEGK (QQQTGG)	ND	ND	ND

C

	Peptide Sequence	Untreated (fmol)	As ₂ O ₃ (fmol)	Difference (fmol)
K65-SUMO2	RCQQCQAEAKCPKLLPCLHTL (QQQTGG)	1.0	6.3	5.3
K160-SUMO2	FLKHEARPL (QQQTGG)	50.5	163.6	113.1
K490-SUMO2	KVIMKESEEGK (QQQTGG)	2.5	2.0	-0.5

Figure 5. Identification of the SUMO switching lysine and the SUMO chain forming lysine on PML

A) FLAG immunoprecipitates from As₂O₃-treated CHO-K1 cells stably expressing FLAG-PML-IV-GFP mutants were immunoblotted with the antibodies indicated. The asterisk indicates a nonspecific band cross-reacting with the FLAG antibody. The data is representative of 2 independent experiments. B) Mass-spectrometry analysis of the SUMO modification status of Lys⁶⁵, Lys¹⁶⁰ and Lys⁴⁹⁰ of PML after As₂O₃ treatment. FLAG-PML-IV was immunoprecipitated from HEK293A cells stably expressing the protein. PML was eluted with 3XFLAG peptide and loaded onto a gel for SDS-PAGE. Five consecutive

slices for each sample in the molecular weight range 70–300 kDa were cut for analysis and synthetic AQUA peptides were used to map and quantitate the SUMO modification sites. Because there were no obvious differences in the distribution of peptides in the individual bands and to simplify data presentation, the individual band quantities were pooled. The data are from a single analysis. C) The same experiment as in (B), except that to enhance peptide recovery, a single gel slice was excised and analyzed through mass-spectrometry. Synthetic AQUA peptides were used to map the SUMO modification sites. The data is an average of two technical replicates. In each experiment, the difference column represents the quantity of As₂O₃-treated minus control peptides.

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Figure 6. E3 ligase activity of SUMO1-RING promotes SUMO2 conjugation on Lys¹⁶⁰

A) Representation of PML and SUMO-PML constructs used to perform *in vitro* reconstitution of SUMO modification triggered by As₂O₃. B) HA-SUMO-RING-B1-B2 constructs carrying a C-terminal His tag were subjected to *in vitro* SUMO2 conjugation reactions with AOS1/UBA2, UBC9 and SUMO2. Mutation of Lys¹⁶⁰ abrogated SUMO2 conjugation when the SUMO1 fusion was used (right panel), but not when the SUMO2 fusion was used (left panel), revealing that SUMO2 attachment does not occur on Lys¹⁶⁰ in the SUMO2 fusion construct. Asterisk indicates cross reactivity of anti-SUMO2 antibody with the high concentration of HA-SUMO1-RING-B1-B2 used in this experiment. The data is representative of two independent experiments. C) and D) FLAG immunoprecipitates from CHO-K1 cells stably expressing FLAG-PML-IV (aa 49-633) mutants and treated with As₂O₃ were immunoblotted with the antibodies indicated. The asterisk indicates a non-specific band cross-reacting with the FLAG antibody. The data in (C) is representative of

three independent experiments while the data in (D) is representative of two independent experiments.

A) We have included a flow chart to aid readers in following the experimental findings and conclusions. B) Under basal conditions, Lys⁶⁵ of PML is conjugated to SUMO2, which does not allow the propagation of the signal. Our data suggest a dynamic situation with constant conjugation and deconjugation of SUMO2. Thus, SENP1-dependent SUMO2 deconjugation counterbalances the SUMO conjugation machinery. The aggregation of PML brought about by As₂O₃ alters PML structure and favors SUMO1 conjugation to Lys⁶⁵ to promote SUMO2/3 conjugation to the chain-forming Lys¹⁶⁰. PML has been depicted as monomer for simplicity.