SUMO deconjugation is required for arsenic-triggered ubiquitylation of PML

Domenico Fasci,1,2 Veronica G. Anania,3 Jennie R. Lill,3 Guy S. Salvesen1*

Acute promyelocytic leukemia is characterized by a chromosomal translocation that produces an oncogenic fusion protein of the retinoic acid receptor α (RARα) and promyelocytic leukemia protein (PML). Arsenic trioxide chemotherapy of this cancer induces the PML moiety to organize nuclear bodies, where the oncprotein 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. On the basis of 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 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 DESI-2 (deSUMOylating isopeptidases 1 and 2), and USP1 (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 by both 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 posttranslational modification of proteins and trigger specific nuclear events in response to cellular stress (11). As2O3 promotes the cross-linking 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 (RING finger protein 4), 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 toward conjugation of PML. To test this hypothesis, we analyzed the activity of endogenous SENPs after As2O3 exposure of human embryonic kidney (HEK) 293A (Fig. 1) and Cos7 (fig. S1) cells by using the


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1Cell Death and Survival Networks Program, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA. 2Graduate School of Biomedical Sciences, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA. 3Department of Protein Chemistry, Genentech Research and Early Development, South San Francisco, CA 94080, USA. *Corresponding author. E-mail: gsalvesen@sanfordburnham.org
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) [Fig. 1 and fig. S1, hemagglutinin (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 used 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 SUMO1 or SUMO2 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 (Chinese hamster ovary–K1) cells, a cell line with low amounts of endogenous PML (17) (Fig. 2, A and B), and in HEK293A cells (fig. S2A). Overexpression of non-deconjugatable SUMO mutants did not alter PML protein abundance (Fig. 2A and fig. S2A). As expected in cells expressing the non-deconjugatable SUMO2-Q90P, global SUMO2 conjugation (all substrates including PML) was enhanced (Fig. 2A and fig. 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 (Fig. 2A and fig. 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 (Fig. 2, A and B, and fig. S2A).

These findings seemed counterintuitive 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 used 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 (fig. S3), confirming that the mechanism is RNF4-dependent. Synthetic 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 (green fluorescent protein)–RNF4 (fig. S4A) and monitored protein abundance after overexpression of the different SUMO2 mutants (fig. 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 decreased in the presence of non-deconjugatable SUMO2 polymers. To test this possibility, we generated trimeric SUMO2 variants using wt or non-deconjugatable mutant (fig. S5A) and evaluated their effect on the processing of SUMO2-modified RanGAP1 by recombinant full-length SENP1 (fig. S5B). We performed the deconjugation reaction in the presence of a fivefold molar excess of the SUMO2 trimers over SENP1, and we did not observe any substantial delay of SUMO2-RanGAP1 processing (fig. S5B), suggesting that SUMO2-Q90P polymers do not sequester SENP1 or affect its activity.

We next evaluated the effect of non-deconjugatable SUMO1 on As2O3-induced ubiquitylation of PML. In cells coexpressing FLAG–PML-IV with non-deconjugatable HA-SUMO1-Q94P, the addition of non-conjugatable mutant together with the addition of SUMO2/3 ubiquitin chains to FLAG–PML-IV after As2O3 treatment was not substantially changed (Fig. 2, C and D, and fig. 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 paralog switch from SUMO2 to SUMO1**

We were faced with the apparent dilemma that enhanced SUMO2 polymerization reportedly drives ubiquitylation of PML...
Fig. 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 HA-SUMO2-Q90P. Data are 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 HA-SUMO1-Q94P. Data are 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 ± SEM. P < 0.05, Student’s two-tailed t test.

SENP1 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, short hairpin RNA (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 increased 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 (Fig. 3A and fig. S6A). The reduction in ubiquitylation was most readily observed in cells transiently transfected with HA-SUMO2 (Fig. 3A) and also seen in cells
with endogenous amounts on SUMO2, validated with an additional shRNA targeting SENP1 (fig. S6A). Reduction of PML ubiquitylation after SENP6 knockdown (fig. S7) was also observed, validating a report that knockdown of SENP6 triggers RNF4 activation and self-degradation (J8). 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 (fig. S6B).

Moreover, knockdown of SENP1 recapitulated the pattern of SUMO modification observed with overexpression of non-deconjugatable SUMO2-Q90P (Fig. 2A and fig. S2A). We observed enhanced basal SUMO2 modification of PML-IV, decreased SUMO2 chain elongation after As2O3 treatment, and reduced SUMO1 conjugation and ubiquitylation (Fig. 3, A and B). In contrast, knockdown of SENP5 did not reduce As2O3-triggered PML ubiquitylation, thus assigning specific SENP isoforms to this mechanism (fig. 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 (Fig. 3A).

As2O3 promotes the transfer of PML proteins from the nucleoplasmic fraction to the nuclear matrix to drive PML aggregation and subsequent SUMOylation (J2, J21). 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 paralog 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 (J21), we next asked whether the activity of SENP1 was required for the previously reported changes (J3) 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 (Fig. 4, A and B), consistent with previous findings (J13). The changes in nuclear body dynamics were reflected in the appearance of high-molecular weight endogenous PML species (Fig. 4C). Knockdown of SENP1 delayed the appearance of high–molecular weight PML forms (Fig. 4C); however, a fraction of endogenous PML proteins persisted after 24 hours.
of As$_2$O$_3$ treatment, in agreement with recent findings (22). Moreover, knockdown of SENP1 led to a significant decrease in the amount of nuclear bodies formed after 1 hour of As$_2$O$_3$ treatment (Fig. 4, A and B). Our findings indicate that the activity of SENP1 is required for formation and stabilization of PML nuclear bodies after As$_2$O$_3$ treatment.

The SUMO2/3 chain-forming lysine in PML is Lys$^{160}$, and the SUMO switching lysine is Lys$^{65}$

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 Lys$^{160}$ 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 As$_2$O$_3$ 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 As$_2$O$_3$-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 As$_2$O$_3$ treatment (fig. S8). SUMO1 knockdown resulted in a strong reduction of SUMO2 conjugation and consequent ubiquitylation of PML after As$_2$O$_3$ treatment (fig. 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 SUMO1 paralog in regulating PML ubiquitylation.

lysine→arginine point mutations. Mutation of residue 160 substantially decreased As$_2$O$_3$-triggered SUMO2/3 PML modification and subsequent ubiquitylation (Fig. 5A). As$_2$O$_3$ treatment still triggered SUMO1 modification of this PML mutant, suggesting that other lysines, most likely Lys$^{65}$ and Lys$^{490}$, were modified by SUMO1 after As$_2$O$_3$ treatment. Mutation of Lys$^{490}$ did not affect ubiquitylation triggered by As$_2$O$_3$ (Fig. 5A), suggesting that modification of this residue played a less crucial role in SUMO-dependent ubiquitylation of PML. Mutation of Lys$^{65}$ slightly decreased ubiquitylation and strongly reduced SUMO2/3 and SUMO1 conjugation of PML (Fig. 5A). This result implied that Lys$^{65}$ could be the SUMO switching lysine that was dynamically modified by SUMO2/3 under basal conditions with a switch to SUMO1 after As$_2$O$_3$ treatment. The strong reduction of SUMO2/3 conjugation observed with this PML mutant prompted us to hypothesize that the switching residue Lys$^{65}$ controls SUMO2/3 addition to the chain-forming Lys$^{160}$.

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 used quantitative mass spectrometry to assess PML obtained from HEK293A cells stably expressing FLAG–PML-IV after As$_2$O$_3$ treatment. To identify SUMO-modified lysines of PML, we immunoprecipitated SUMO-modified PML from cells treated or untreated with As$_2$O$_3$ 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 Lys$^{490}$. To quantify the degree of SUMOylation, we obtained synthetic branched AQUA peptides corresponding to the chymotryptic or tryptic/chymotryptic peptides encompassing Lys$^{65}$, Lys$^{160}$, or

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**Fig. 4.** SENP1 activity is required for PML nuclear body formation after As$_2$O$_3$ treatment. (A) HEK293A cells transfected with an shRNA for SENP1 or empty pLKO vector were selected with puromycin, plated on coverslips, and treated with As$_2$O$_3$. Changes in PML nuclear body formation were assessed through immunofluorescence using an antibody specific for PML. Scale bars, 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 hour after As$_2$O$_3$ stimulation (P < 0.0001). (C) Western blot analysis of whole-cell lysates obtained from the samples used for immunofluorescence with the antibodies indicated. Data are representative of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Lys<sup>490</sup> 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 (Fig. 5, B and C).

We observed equivalent SUMO2/3 modification of Lys<sup>490</sup> in the untreated and As<sub>2</sub>O<sub>3</sub>-treated sample (Fig. 5C), suggesting that modification of this residue in PML does not play a crucial role in the As<sub>2</sub>O<sub>3</sub> response. We noted an increase in SUMO2/3 addition to Lys<sup>65</sup> and a much more substantial increase at Lys<sup>160</sup> after As<sub>2</sub>O<sub>3</sub> treatment (Fig. 5, B and C). This result agreed with the data obtained with the Lys→Arg mutants and demonstrated that Lys<sup>160</sup> was the major SUMO2/3 modification site of PML triggered by As<sub>2</sub>O<sub>3</sub>, providing further evidence that Lys<sup>65</sup> 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 Lys<sup>65</sup> 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 As<sub>2</sub>O<sub>3</sub> enhanced both SUMO2/3 and SUMO1 modification of Lys<sup>65</sup> 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 Lys<sup>65</sup>.

SUMO1 modification of the PML RING domain promotes SUMO2 conjugation to Lys<sup>160</sup>

The question arising from our findings was why SUMO1 modification of PML was required for SUMO-dependent ubiquitylation of the protein triggered by As<sub>2</sub>O<sub>3</sub> treatment. We hypothesized that SUMO1 modification of Lys<sup>65</sup> was required to promote SUMO2 modification on Lys<sup>160</sup>, and to test this hypothesis, we reconstituted the SUMO modification of PML in vitro. We simulated SUMO modification of Lys<sup>65</sup> by fusing the different SUMO paralogs to the N terminus of the RING domain of PML (residue 49; fig. S9 and Fig. 6A). With these constructs, we tested the requirements for SUMO conjugation to Lys<sup>160</sup>. In the presence of the conjugating enzymes, HA-SUMO1-RING-B1-B2 was conjugated to SUMO2, which was selective for Lys<sup>160</sup> (Fig. 6B). In contrast, SUMO2 was conjugated to wt or K160R HA-SUMO2-RING-B1-B2 constructs at similar extents, demonstrating that SUMO1, not SUMO2, specifically directed the conjugation of SUMO2 to Lys<sup>160</sup> (Fig. 6B). Presumably, SUMO2 was conjugated to another lysine, possibly the SUMO2 moiety in the K160R construct, most likely at the chain extension site Lys<sup>41</sup> (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 (Fig. 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 polyproline-rich domain (amino acids 1 to 48) from full-length PML-IV did not substantially affect As2O3-triggered ubiquitylation (Fig. 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 (Fig. 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 (Fig. 6D). These data are consistent with a requirement of the conjugation SUMO1, but not

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**Fig. 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. The asterisk indicates cross-reactivity of anti-SUMO2 antibody with the high concentration of HA-SUMO1-RING-B1-B2 used in this experiment. Data are representative of two independent experiments. (C and D) FLAG immunoprecipitates from CHO-K1 cells stably expressing FLAG–PML-IV (amino acids 49 to 633) mutants and treated with As2O3 were immunoblotted with the antibodies indicated. The asterisk indicates a nonspecific band cross-reacting with the FLAG antibody. The data in (C) are representative of three independent experiments, whereas the data in (D) are representative of two independent experiments.

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 (Fig. 7A). We present evidence for a deconjugation-controlled SUMO signal. In our working model (Fig. 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 (Fig. 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 in both the in vitro and cellular experiments do not support this hypothesis. The SUMO2-K11R construct does not appear to behave like SUMO1 (fig. S10 and Fig. 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, argue 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 cross-linking of PML induced by As2O3 alters the SUMO conjugation/deconjugation balance. Second, because As2O3-induced PML cross-linking may protect the substrate from SUMO deconjugation, SUMO1 modification of PML Lys65 would now promote the synthesis of SUMO2/3 chains on Lys160 (Fig. 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 cross-linking coupled with SUMO1 conjugation may also be required to stabilize the PML nuclear bodies, preventing prompt SUMO

![Diagram](https://example.com/diagram.png)
deconjugation and premature disassembly. Future studies will be needed to
demonstrate the exact nature of the response of PML to As$_2$O$_3$, and
these could include overcoming the technical challenge of purifying intact
PML nuclear bodies and exploring the role of different PML splicing var-
iants in propagating the SUMO-dependent ubiquitin signal.

The reversible modification of Lys$_{65}$ leading to modification of Lys$_{160}$
seems like a complex way to organize cell signaling, and in 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 Lys$_{65}$ of PML dom-
ninates unstimulated conditions, but upon As$_2$O$_3$ 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
Lys$_{65}$ and subsequent SUMO2/3 chain formation at Lys$_{160}$. Thus, the sub-
strate 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 (30) and of molecular
noise in stochastic bacterial gene activation networks (31). 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 #AF230406) was amplified from a
human keratinocyte complementary DNA (cDNA) library. The polymerase
chain reaction (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 mutagen-
sis using primers carrying the specific mutation. RanGAP1ΔN in pET28b,
proSUMO2 in pET28, SUMO1, SUMO2, and SUMO2-Q90P constructs
cloned into pcDNA3 with an N-terminal HA tag were previously described
(7, 32). SUMO1-Q94P was generated using a reverse primer carrying the
specific mutation. Full-length SENP1 construct (7) was subcloned in
pGEX4T1. pTE1E2S2 was provided by H. Saitoh (Department of Bio-
logical Sciences, Kumamoto University) (33). pTE1E2S2-Q90P was gen-
erated with site-directed mutagenesis using a primer carrying the specific
mutation. pTYB2-αHA-SUMO2 was provided by W. Wilkinson (Depart-
ment of Biochemistry, Emory University, Atlanta, GA) (34). Mouse RN4F
and RN4F-C177S constructs cloned in pGFP-C1 were provided by T. Hunter
(Salk Institute for Biological Studies, La Jolla, CA) (35). PML RING-B1-B2
(aminic acids 49 to 236) and SUMO-RING-B1-B2 (SUMO1 amino acids 1 to
97 + PML amino acids 49 to 236 or SUMO2 amino acids 1 to 97 + PML
amino acids 49 to 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 provided by
F. Melchior (Universität Heidelberg, Heidelberg, Germany) (36). FLAG–
SUMO–PML-IV fusion mutants were generated with overlapping PCR,
fusing SUMO1 (amino acids 1 to 97) or SUMO2 (amino acids 1 to 93) with
PML-IV (amino acids 49 to 633); FLAG tag was introduced at the N termi-
nus of each construct with the forward primer; and the last glycine of each
SUMO paralog was mutated to alanine to prevent SUMO removal. The en-
tire 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 (CellGro) supplemented with 10% heat-inactivated fetal bovine
serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 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, penicillin
(100 U/ml), streptomycin (100 μg/ml), and 2 mM l-glutamine. Stable cell
lines expressing GFP-RN4F, FLAG–PML-IV–GFP wt and mutants, FLAG–
PML-IV wt and mutants, and FLAG SUMO–PML-IV fusion mutants
cloned, respectively, in pEGFP-C1, pEGFP-N2, and pcDNA3 were ob-
tained by transfecting HEK293A or CHO-K1 with NanoJuice Transfection
Reagent (Novagen) according to the manufacturer’s instructions. Trans-
fected cells were selected for 2 weeks with G418 (500 μg/ml) and propagated
after selection. As$_2$O$_3$ (Sigma) was dissolved in 1 M NaOH, diluted, and
pH-adjusted to get a stock concentration of 5 mM (pH 8). As$_2$O$_3$ 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 trans-
fected using the corresponding shRNA with NanoJuice Transfection Re-
agent (Novagen) according to the manufacturer’s instructions, selected for
72 hours with puromycin (1 μg/ml), and treated accordingly.

Immunoblotting and immunoprecipitation

Treated cells were scraped and washed twice in ice-cold phosphate-buffered
saline (PBS), and pellets were frozen at −80 °C until all the time points were
collected. Cell pellets were resuspended in radioimmunoprecipitation assay
buffer [50 mM tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 0.5% sodium
doxycholate, 1% Triton X-100 (TX100), and 0.5% SDS], supplemented
with 50 mM NEM (Pierce) and protease inhibitors [trans-epoxyisuccinyl-1-
leucylamido(4-guanidino) butane, 3,4-dichloroisocoumarin, leupeptin, and
MG-132; all 10 μM]. After resuspension, lysates were boiled for 5 min
extracted from PML proteins from the nuclear matrix and disrupt protein
complexes, diluted 1:3 in TBS (tris-buffered saline) with 1% TX100, and
passed through a 27-gauge 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 im-
munoprecipitation. FLAG immunoprecipitation was performed by incu-
bating 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 (LI-COR), and
signal was quantified using the Odyssey system. Statistical analysis was per-
formed using GraphPad Prism software (GraphPad Software Inc.).

Immunofluorescence

Cells were grown on coverslips, treated, washed twice with PBS, and fixed
for 20 min in 4% paraformaldehyde and 2% sucrose in PBS at room tem-
perature. Permeabilization and blocking were performed at room tempera-
ture for 30 min in PBS, 0.4% TX100, and 2% BSA (bovine serum albumin).
Staining with primary antibodies was performed for 1 hour at room tem-
perature 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, and
1% BSA and stained with secondary antibodies conjugated with Alexa Fluor
568 (Life Technologies). The coverslips were mounted for imaging with
Vectashield containing 4’,6-diamidino-2-phenylindole. Images were

Downloaded from http://stke.sciencemag.org/ on February 5, 2021
acquired using an LSM 710 NLO Zeiss multiphoton laser point scanning confocal microscope. The 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.).

**Mass spectrometry analysis of the SUMOylation status of Lys160, Lys166, and Lys488 in PML**

HEK293A cells stably expressing FLAG–PML-IV were grown and treated with 2 μM 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 3X FLAG peptide (5 mg/ml) and incubation on ice. Eluted samples were subjected to Laemmli SDS-PAGE, and PML material was excised from the gel as a single slice. Gel pieces were diced into 1-mm cubes and destained in 50 mM ammonium bicarbonate (Ambic) in 50% (v/v) acetonitrile (ACN) for 15 min or until clear. Gel pieces were dehydrated with 30 μl of 100% ACN for 5 min, the liquid was removed, and gel pieces were rehydrated in 5 mM dithiothreitol and incubated at 50 °C for 60 min. Gel pieces were again dehydrated in 100% ACN, the 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 μM sequencing-grade chymotrypsin (Pierce Biotechnology) and 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 of 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 Lys190. Arginine or lysine carried the isotopic label adding 25% excess mass to these residues. All reactions were performed in triplicate.

Samples were injected in duplicate through an autosampler onto a nanoAcquity UPLC (Waters) over a 2-hour gradient and analyzed on- line through nanospray ionization into an LTQ Orbitrap Elite mass spectrometer operated in data-dependent mode. Full mass spectrometry transitions were selected for fragmentation in the ion trap. Reactants were injected as a 20 μl of reactions containing 2 μM substrate, 5 μM SUMO2, 300 nM AO11, 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 Biotechnology), anti-FLAG (M2, Sigma), anti–β-tubulin (ab6046, Abcam), anti-GAPDH (#2118, Cell Signalling), anti-GFP (D5.1 XP, Cell Signalling), anti-HA for Western blotting (clone 16B12, Covance), anti-ubiquitin (clone FK2, Enzo Life Sciences; whole antiserum U5379, Sigma), and anti-SUMO1 and anti-SUMO2 antisera were provided by H. McBride (McGill University, Montreal, Canada) (37). Anti-SENP1 and anti-SENP6 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 to 643 for antigen for SENP1 and residues 628 to 1112 for SENP6, followed by affinity purification. SENP5 antiserum was provided by H. McBride (McGill University, Montreal, Canada) (37). Anti-SENP5 activity is not required for SUMO-dependent ubiquitylation triggered by As2O3 treatment. SENP5 antiserum was provided by H. McBride (McGill University, Montreal, Canada) (37). Anti-SENP5 activity is not required for SUMO-dependent ubiquitylation triggered by As2O3 treatment. Anti-SENP5 activity is not required for SUMO-dependent ubiquitylation triggered by As2O3 treatment.

**Protein purification and in vitro SUMOylation**

All recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) (Novagen). Protein expression was induced at OD600 (optical density at 600 nm) = 0.6 with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 5 hours at 25 °C. PML constructs (amino acids 49 to 236) were expressed in the presence of 100 μM ZnCl2. To generate SUMO2-modified His-RanGAP1ΔN, we coexpressed the RanGAP1 construct with pTET1E2S2. Tri-SUMO2-wt and Q90P were generated, coexpressing proSUMO2-His tagged with pTET1E2S2 or pTET1E2S2-Q90P. His-tagged proteins were purified using Chelating Sepharose Fast Flow (GE) charged with 100 mM NiSO4 and eluted with a 20 to 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). Glutathione S-transferase–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 (36). HA-SUMO2-vme was prepared using a procedure described in the literature (34). Protein purity was evaluated through SDS-PAGE, and concentration was determined through BCA Protein Assay (Pierce). In vitro SUMOylation was performed in 20 μl of 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.

**REFERENCES AND NOTES**


**SUPPLEMENTARY MATERIALS**

www.sciencesignaling.org/cgi/content/full/8/380/ra56/DC1

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. The SUMO2-K11R-RING construct does not mimic the SUMO1-RING construct.

Fig. S6. SENP1 activity is not affected by a non-deconjugatable SUMO2 trimer.

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

Fig. S8. SENP1 is required for the ubiquitylation of PML-IV after As2O3 treatment.

Fig. S9. Purified proteins used for in vitro SUMOylation. Protein purification and in vitro SUMOylation.

Fig. S10. The SENP5 activity is not required for SUMO-dependent ubiquitylation triggered by As2O3 treatment.

Table S1. shRNA sequences.

Table S2. Summary of synthetic isotopic peptides.


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SUMO deconjugation is required for arsenic-triggered ubiquitylation of PML
Domenico Fasci, Veronica G. Anania, Jennie R. Lill and Guy S. Salvesen

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SUMO switching for degradation
Promyelocytic leukemia protein (PML) organizes various proteins into structures called PML nuclear bodies. Acute promyelocytic leukemia is caused by a fusion protein consisting of PML and the transcription factor RAR α. Degradation of this oncoprotein can be induced by arsenic trioxide chemotherapy through modification of PML by SUMO1, SUMO2, and ubiquitin. Fasci et al. found that in untreated cells, SUMO2 was constantly added to Lys65 in PML and constantly removed from this residue by the deconjugating enzyme SENP1. In cells exposed to arsenic trioxide, SUMO1, rather than SUMO2, was conjugated to Lys65, which led to the formation of SUMO2 chains on a different residue, Lys65, that in turn triggered the ubiquitylation of PML, as well as reorganization of PML nuclear bodies. These results further define how arsenic trioxide induces the degradation of the RARα-PML oncoprotein, by promoting an exchange of SUMO paralogs on a “switch” residue that stimulates SUMO modification on a “chain” residue.