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Arsenic trioxide stimulates SUMO-2/3 modification leading to RNF4-dependent proteolytic targeting of PML

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Abstract **We have recently reported that poly-SUMO-2/3 conjugates are subject to a ubiquitin-dependent proteolytic control in human cells. Here we show that arsenic trioxide (ATO) increases SUMO-2/3 modification of promyelocytic leukemia (PML) leading to its subsequent ubiquitylation *in vivo*. The SUMO-binding ubiquitin ligase RNF4 mediates this modification and causes disruption of PML nuclear bodies upon treatment with ATO. Reconstitution of SUMO-dependent ubiquitylation of PML by RNF4 *in vitro* and in a yeast trans vivo system revealed a preference of RNF4 for chain forming SUMOs. Polysumoylation of PML in response to ATO thus leads to its recognition and ubiquitylation by RNF4.**

Keywords: Small ubiquitin-like modifier; Ubiquitin; Relatively interesting new gene finger; Protein degradation; Promyelocytic leukemia

1. Introduction

The role of certain poly-ubiquitin chains as signals for degradation of proteins by the 26S proteasome is well established [1]. While in mammalian cells there are examples where modification with small ubiquitin-related modifier isoform 1 (SUMO-1) stabilizes a protein by antagonizing its ubiquitylation [2], recent studies in yeast have established that SUMO modification can also serve as a proteolytic targeting signal that is recognized by ubiquitin ligases for SUMO conjugates (ULS) [3–7]. These ULS proteins, which are characterized by a relatively interesting new gene (RING) domain and short SUMO interaction motifs (SIMs), bind to polysumoylated proteins and prevent their accumulation. Mutations in yeast ULS genes can be complemented by the human ubiquitin ligase RNF4/SNURF indicating that it is a human ULS [8,4–6]. RNF4 interacts with PML [9] and may thus be involved in a proteolytic control of PML. Experiments in human cells showed that mainly high molecular weight conjugates of the nearly identical chain forming SUMO-2 and SUMO-3 isoforms are subject to a proteolytic control by the proteasome. In contrast to SUMO-1, conjugates of SUMO-2/3 isoforms have been shown to be induced by heat, oxidative or osmotic stress [10].

Sumoylation of the human PML protein is necessary for the formation of PML-NBs (reviewed in [11]). The normal PML protein as well as the oncogenic fusion protein between PML and the retinoic acid receptor α (RARα), which causes acute promyelocytic leukemia (APL), are degraded in response to arsenic trioxide (ATO, reviewed in [12]). Under these conditions sumoylation at lysine 160 precedes degradation [13]. In this study, we have investigated the mechanism that leads to PML downregulation in response to ATO treatment. We demonstrate a direct link between ATO-induced SUMO-2/3 modification of PML and its RNF4-mediated and ubiquitin-dependent degradation by the proteasome.

2. Materials and methods

2.1. Stable cell lines inducibly expressing His-tagged SUMO isoforms

SUMO-1, -2 and -3 were cloned with an N-terminal MRGS-His_6 epitope into a pCDNA4/TO/myc-His B. T-REx HeLa cells (Invitrogen) were grown in EMEM (Gibco), 2 mM glutamine, 1% non-essential amino acids, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml blasticidin (InvivoGen) and stably transfected with Gene Juice (Novagen). Selection was carried out with 150 μg/ml zeocin (InvivoGen) and individual colonies were subcloned, expanded and screened for similar induction properties.

2.2. Analysis of SUMO conjugates in stable His_6-SUMO expressing cells

Cells were transiently transfected, after 9 h induced with 1 μg/ml doxycyclin (Fluka) and 15 h later treated with 1 μM ATO (Alfa Aesar) and/or 20 μM MG132 in DMSO (Biomol) for 6 and 3 h, respectively. Nickel-nitrilo triacetic acid (Ni-NTA) affinity purifications were performed with $\sim 5 \times 10^6$ cells in urea buffer as described [6]. In some experiments, the eluates were used for a subsequent FLAG pull-down. For direct FLAG pull-downs, whole cell lysates from $\sim 5 \times 10^6$ cells were prepared under alkaline conditions and subjected to immunoprecipitations as described [6].

2.3. Protein purification and in vitro ubiquitylation

SUMO-modified PML proteins were purified from an *Escherichia coli in vivo* sumoylation system, in which N-terminally glutathione

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Abbreviations: ATO, arsenic trioxide; Ni-NTA, nickel-nitrilo triacetic acid; GSH, glutathione; GST, glutathione *S*-transferase; His_6, 6×His tag; NB, nuclear body; PML, promyelocytic leukemia; RING, relatively interesting new gene; RNF4, RING finger protein 4; SAE, SUMO activating enzyme; SIM, SUMO interaction motif; SUMO, small ubiquitin-related modifier; UBC9, SUMO conjugating enzyme; ULS, ubiquitin ligase for SUMO conjugates

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S-transferase (GST)-tagged PML 50-179 and untagged PIAS4 were expressed from pET-Duet1, His_6-SUMO-1 or -3 and untagged SUMO conjugating enzyme (UBC9) from pRSF-Duet1, and human His_6-SAE1 and untagged SAE2 from pACYC-Duet1 (all Novagen). Expression in BL21 (DE3) cells was induced by 0.1 mM IPTG at 30 °C. His_6-SUMO-modified PML was isolated by consecutive glutathione (GSH)-sepharose and Ni-NTA affinity purifications, TEV protease cleavage of the GST-tag and gel filtration. Unmodified PML and RNF4 were isolated by GSH-sepharose affinity purification, TEV cleavage and gel filtration. RNF4-dependent ubiquitylation reactions were carried out for 7 h at 30 °C in 40 mM Tris/HCl, pH 7.5, 67 mM KCl, 4 mM $MgCl_2$, 5 mM ATP, 200 μg/ml BSA, 2 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 200 μM pefabloc and contained 3 μg/ml UBE1 (Biomol), 3 μg/ml UBCH5b, 300 μg/ml ubiquitin (Biomol), 100 μg/ml substrate and 30 μg/ml RNF4 where indicated.

2.4. Immunofluorescence and antibodies

Cells were grown on cover slips and fixed in PBS with 3% paraformaldehyde. After permeabilization in PBS with 0.1% saponin (Sigma–Aldrich), cells were blocked in PBS, 3% BSA, 0.2% saponin. Primary and Alexa-labeled secondary antibodies (Invitrogen) were applied in blocking buffer. Cover slips were embedded in ProLong Gold antifade (Invitrogen) and examined using a Zeiss Axioplan2 fluorescence microscope. DsRed-Mito (Clontech) was used as a transfection control. Proteins and epitope tags were detected with the following antibodies: SUMO-1: anti-GMP1 (Zymed), SUMO-2/3: ab22654 (Abcam), Smt3: rabbit serum [6], PML: 5E10 [14] and sc-5621 (Santa Cruz), His-tag: 34650 or 34660 (Qiagen), FLAG: M2 or F7425 (Sigma–Aldrich), HA: 3F10 (Roche), Myc: 9E10 (Sigma–Aldrich), T7: T7-HRP, 69048 (Merck).

2.5. Experiments in Saccharomyces cerevisiae

The yeast strains JD47-13C (wt) and a 2 μm/*LEU2*-based plasmid (pKG87) expressing RNF4 from P_{CUP1} were described previously [6]. N-terminally T7-tagged PML 1–522 was expressed from P_{GAL1} in a CEN/URA3-based plasmid (pKG117), a derivative of YCplac33 [15]. YKU25-1 is a spontaneous temperature sensitive *uba2* derivative of JD47-13C. The mutation was identified as a suppressor of *ulp2*Δ (kind gift of K. Uzunova). Cells were grown in synthetic media containing 2% galactose and 100 μM $CuSO_4$. Preparation of extracts and analysis by immunoblotting and pull-downs of T7-PML from extracts prepared was performed with T7-resin (Merck) essentially as described above for FLAG-tagged proteins.

3. Results and discussion

We have recently shown that SUMO-2/3 conjugates are targeted for proteasomal degradation by ubiquitylation [6]. Based on these data, we suggested that the observed ATO-induced degradation of PML following its sumoylation may involve ULS-mediated targeting [13,6]. In order to test this hypothesis, we first analyzed the effect of ATO on the conjugatable SUMO isoforms in human cells. Similar to osmotic and oxidative stress [10], ATO resulted in a significant increase in SUMO-2/3 conjugates, which were accumulating to very high levels upon inhibition of the proteasome (Fig. 1A). SUMO-1 conjugates, in contrast, were much less affected by these treatments. Consistent with previous studies [11], we observed upregulation of modified endogenous PML in untreated as well as in interferon-γ-treated cells upon treatment with ATO (Fig 1B). These forms were apparently stabilized upon inhibition of the proteasome. In order to analyze the modification of sumoylated PML in more detail, we established inducible cell lines expressing His_6-tagged versions of SUMO-1, -2 or -3. We then transfected these cell lines with a FLAG-tagged truncated version of splice form PML11 (residues 1–522), which comprises the regulatory regions present in most PML isoforms and in the PML-RAR-α fusion protein, namely the TRIM domain, three sumoylation sites and a recently described degron around the SIM [16]. Treatment with ATO resulted in the appearance of several distinct higher molecular weight forms of sumoylated PML between 100 and 170 kDa in the transfectants coexpressing FLAG-PML 1–522 and His_6-SUMO-2 or -3, but not in the His_6-SUMO-1 coexpressing cells (Fig. 1C and D). Similar results were obtained for FLAG-tagged full length isoforms 6 and 11 (data not shown). In contrast to the situation for endogenous PML, inhibition of the proteasome in the transfectants had no detectable effect on modified FLAG-PML 1–522 (Fig. 1C). Likely reasons are the high levels of FLAG-PML together with the strong depletion of ubiquitin upon proteasome inhibition (see also Fig. 1E). The upregulation of SUMO-2/3 modified forms of FLAG-tagged PML 1–522 occurred not only after ATO treatment but also upon oxidative and osmotic stress (data not shown). Together, these results suggested that upregulation of SUMO-2/3 conjugates and the degradation of PML in response to ATO are linked processes. The high molecular weight forms of His_6-SUMO-2/3 conjugated FLAG-PML were absent in case of the K160R mutant (data not shown), consistent with the observed resistance of this mutant towards ATO-dependent degradation [13].

We have previously shown that SUMO-ubiquitin hybrid conjugates exist in yeast and human cells [6]. Sequential Ni-NTA and anti-FLAG affinity purifications from His_6-SUMO-2 expressing cells co-transfected with FLAG-PML 1–522 and HA-ubiquitin revealed that high molecular weight hybrid conjugates of PML occur *in vivo* upon ATO treatment (Fig. 1E). This indicates that such conjugates represent intermediates on the way towards proteasomal ATO-induced degradation of PML.

Since PML can be sumoylated in yeast cells [17], we have tested the effect of the potential human ULS protein RNF4 on levels of SUMO conjugates and on PML (Fig. 2A and B). Expression of T7-tagged PML 1–522 led to the appearance of several bands corresponding to unmodified and yeast SUMO-conjugated PML (compare anti-T7 and anti-Smt3 blots). The slower migrating bands were absent from a strain with an impaired SUMO-activating enzyme (*uba2-ts*) [18] (Fig. 2B, left panel). We confirmed that these bands represent sumoylated PML by detection with anti-Smt3 after immunoprecipitation of T7-PML (Fig. 2B, middle and right panel). In the presence of RNF4, the slower migrating bands were diminished indicating that RNF4 targets Smt3/SUMO-modified PML to degradation in *S. cerevisiae*. Moreover, the expression of RNF4 alone decreased the levels of Smt3 conjugates of endogenous yeast proteins (Fig. 2A).

In order to directly show that RNF4 mediates a SUMO-dependent ubiquitylation of PML, we reconstituted this reaction *in vitro*. To achieve this, we set up reactions using recombinant ubiquitin, UBE1, UBCH5b, RNF4 and PML 50–179 produced in *E. coli*. PML modified with His_6-SUMO-1 or -3 was produced in *E. coli* cells expressing sumoylation enzymes. While RNF4 failed to mediate ubiquitylation of unmodified PML, we detected an RNF4-dependent ubiquitylation of PML modified with SUMO-1 and -3. Both types of SUMO-modified PML were ubiquitylated in an RNF4-dependent manner but His_6-SUMO-3 modified PML was ubiquitylated more efficiently (Fig. 2C). In contrast, ubiquitylation of linear GST-SUMO-1 or -3 fusion proteins as well as of RNF4 itself

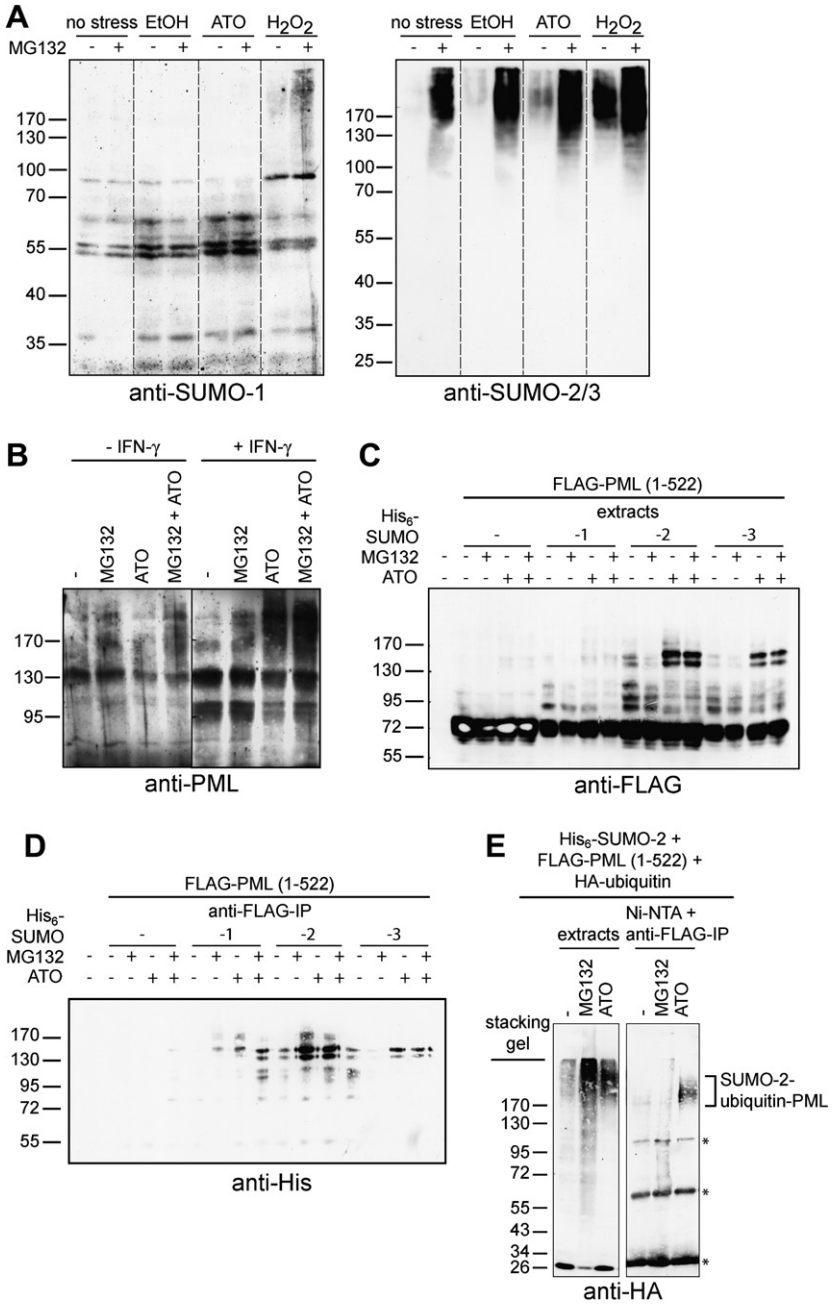

Fig. 1. SUMO-dependent ubiquitylation and degradation of PML induced by ATO. (A) Accumulation of SUMO-2/3 conjugates by ATO and other stress factors. Whole cell extracts of HeLa cells treated with either 7% ethanol for 20 min, 1 μM ATO for 1 h or 100 mM H_2O_2 for 20 min and with or without MG132 prior to stress were analyzed by Western blotting against SUMO-1 (left panel) and SUMO-2/3 (right panel). (B) Degradation of endogenous PML by ATO. Whole cell extracts of untreated (left panel) or interferon-γ stimulated HeLa cells (right panel, 200 U/ml, 24 h) were analyzed by anti-PML Western blotting. (C) Accumulation of high molecular weight forms of PML after treatment with ATO. Anti-FLAG Western blot of whole cell extracts from stably transfected cells expressing His_6-SUMO and transiently transfected with FLAG-PML 1–522 were analyzed by anti-His Western blotting after treatment with and without ATO and MG132. (D) Stimulation of His_6-SUMO-2/3 conjugation of PML by ATO. Anti-His Western blot of anti-FLAG pull-down from cells after treatment as in C. (E) Formation of hybrid SUMO-ubiquitin conjugates of PML after ATO treatment. Anti-HA Western blotting of sequential Ni-NTA and anti-FLAG pull-downs from stable His_6-SUMO-2 cells transiently transfected with FLAG-PML 1–522 and HA-ubiquitin after treatment with MG132 or ATO. Anti-FLAG IgG bands are marked with asterisks.

was negligible under these conditions providing additional evidence for the specificity of the reaction (Fig. 2C and Supplementary Fig. 1). The preference of RNF4, which carries several SIMs, for SUMO-3-modified PML is likely to be due to a higher avidity for SUMO chains as we have observed for the yeast ULS protein Hex3/Slx8 [6].

To test whether RNF4 is involved in the degradation of PML in response to ATO in human cells, myc-tagged RNF4 and FLAG-tagged PML 1–522 were transfected into His_6-SUMO-2 expressing cells and treated with ATO and/or the proteasome inhibitor MG132 (Fig. 2D). As previously described, overexpressed RNF4 and PML co-localize in

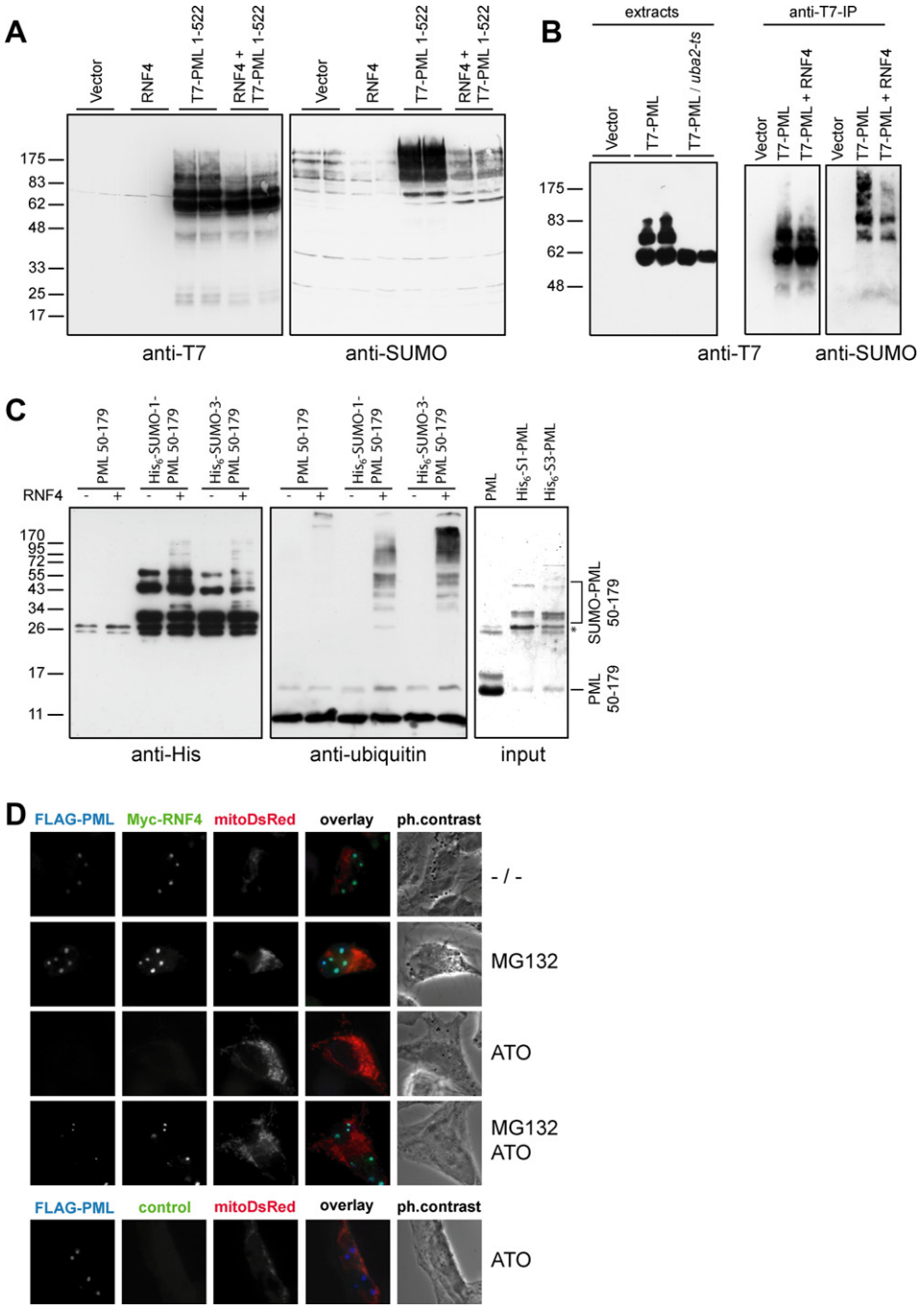

Fig. 2. The human ULS protein RNF4 targets sumoylated PML. (A) Overexpression of RNF4 reduces the levels of endogenous SMT3/SUMO conjugates (right panel, in duplicates) and of sumoylated PML in *Saccharomyces cerevisiae* (left panel, in duplicates). (B) Sumoylation of T7-PML 1–522 in *S. cerevisiae* is absent in *uba2-ts* cells (left panel, in duplicates). Sumoylated forms were detected by anti-Smt3 blotting after pull-down of T7-PML (right panel). (C) RNF4-dependent ubiquitylation of SUMO-modified PML *in vitro*. Unmodified, His_6-SUMO-1- and His_6-SUMO-3-modified PML 50-179 were generated in *Escherichia coli* and purified by liquid chromatography (right panel). RNF4-dependent ubiquitylation was analyzed by SDS–PAGE followed by anti-His (left panel) and anti-ubiquitin (middle panel) Western blotting. Residual His-tagged TEV protease is marked with an asterisk. (D) RNF4 and ATO act synergistically in dispersal of PML-NBs. Cells expressing His_6-SUMO-2 were transiently transfected and stained for FLAG-PML 1–522 (blue) and Myc-RNF4 (green) after treatment with ATO and MG132. Mito-DsRed was used as a transfection control (red). The bottom panel shows a control without transfection of Myc-RNF4.

PML-NBs [9]. In presence of MG132, these PML-NBs increased in size and number. After addition of ATO hardly any PML-NBs were detectable. When both MG132 and ATO were added, the cells resembled those not treated with either compound. ATO treatment of cells overexpressing PML alone did not lead to PML-NB dispersal.

In summary our data confirm the proposed function of RNF4 as a ULS protein and identify PML as its first

mammalian substrate. Specifically, we show that RNF4 mediates a proteolytic control of PML in response to ATO. The molecular target of ATO, however, is still unknown, as it may interfere with multiple pathways. We found that ATO treatment increases the amount of SUMO-2/3 conjugates to a similar extent as described for stress factors such as oxidative stress [10], and our *in vitro* assay demonstrates a preference of RNF4 for SUMO-3 over SUMO-1 modified substrates. Thus, increased conjugation of SUMO-2/3 or an inhibition of its deconjugation could be sufficient to activate RNF4-dependent degradation of PML. In addition, ATO might trigger signaling events such as the described phosphorylation of PML, which has been shown to precede sumoylation and degradation of PML [19,16]. While this manuscript was in preparation, two other studies appeared that support our view that RNF4 is a ULS that mediates proteolytic targeting of sumoylated PML [20,21].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.08.008.

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