Identification of Three Major Sentrinization Sites in PML*

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Acute promyelocytic leukemia arises following a reciprocal chromosome translocation t(15;17), which generates PML-retinoic acid receptor α fusion proteins (PML-RARα). We have shown previously that wild type PML, but not PML-RARα, is covalently modified by the sentrin family of ubiquitin-like proteins (Kamitani, T., Nguyen, H. F., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. H. (1998) J. Biol. Chem. 273, 3117–3120). To understand the mechanisms underlying the differential sentrinization of PML versus PML-RARα, extensive mutational analysis was carried out to determine which Lys residues are sentrinized. We show that Lys65 in the RING finger domain, Lys160 in the B1 Box, and Lys490 in the nuclear localization signal contributes three major sentrinization sites. The PML mutant with Lys to Arg substitutions in all three sites is expressed normally, but cannot be sentrinized. Furthermore, the triple substitution mutant is localized predominantly to the nucleoplasm, in contrast to wild type PML, which is localized to the nuclear bodies. Thus, sentrinization of PML, in the context of the RING finger and the B1 box, regulates nuclear body formation. Furthermore, we showed that sentrinization of PML-RARα could be restored by overexpression of sentrin, but not by retinoic acid treatment. These studies provide novel insight into the pathobiology of acute promyelocytic leukemia and the sentrinization pathway.

Acute promyelocytic leukemia (APL) is characterized by the presence of a balanced translocation from the long arm of chromosome 17 to the long arm of chromosome 15 (t(15q–; 17q+)), which is present in 70–100% of patients (1). This translocation fuses the PML gene, located on chromosome 15, to the retinoic acid receptor α (RARα) gene located on chromosome 17. Two different types of chromosome break points within the PML gene result in the production of two different PML-RARα fusion products, referred to as the A type and the B type, or the L and the S type (2–6). PML-RARα-A contains the entire NH2-terminal 552 amino acids of PML fused to RARα (amino acids 61–462), whereas PML-RARα-B contains the NH2-terminal 394 amino acids of PML fused to RARα (amino acids 61–462). Thus, most of the functional domains of PML and RARα are preserved in the fusion proteins. The fusion proteins, while typically responsive to retinoic acid, display both cell type- and promoter-specific differences from the wild type RARα (3–5). Overexpression of PML-RARα in U937 cells prevents vitamin D3 and transforming growth factor β-induced differentiation, but enhances sensitivity to retinoic acid (7). PML-RARα also reduces serum starvation-induced apoptosis in U937 cells (7). The coiled-coil region, but not the RING finger domain, of PML is involved in the dimerization of PML with PML-RARα (5) and is required for PML-RARα-induced differentiation block (8). PML-RARα can also dimerize with RARα and RXR (9). Thus, PML-RARα was postulated to function as a dominant negative protein by sequestering and interfering with the function of PML, RARα, and RXR.

In normal cells, PML is localized to a discrete subnuclear compartment called the nuclear body (10, 11) or PML oncogenic domain (12). The nuclear body has also been called nuclear domain 10 (ND10) or Kr body (13). In addition to PML, there are at least five other proteins that have been reported to localize to nuclear bodies. They include Sp100 (14), Sp140 (15), NDP52 (16), Finb (17), and PLZF (18). Interestingly, PLZF-RARα fusion protein has also been reported in a rare form of APL (19). The function of the nuclear body is unknown. The periphery of the nuclear body has been shown to be the preferred site where the early steps of transcription and replication of three DNA virus families take place (20). In APL cells, the nuclear bodies were dispersed into a microspeckle pattern (10–12); however, upon treatment with retinoic acid, reformation of the nuclear body occurred and APL cells differentiated into granulocytes. Thus, the regulation of nuclear body formation provides an important insight into the pathogenesis of APL.

Our laboratory has recently demonstrated that PML, but not PML-RARα fusion proteins, is covalently modified by the sentrin family of ubiquitin-like proteins (21). Two other laboratories have also independently reported modification of PML by sentrin or sentrin-related proteins (22, 23). Furthermore, sentrinization of PML may regulate its localization in the nucleoplasm or in the nuclear matrix, which contains the nuclear body (22). Identification of the sentrin modification sites in PML will be very useful in further understanding the role of sentrinization in the pathogenesis of APL in particular and the biology of sentrinization in general.

Sentrin was discovered in our laboratory in a yeast two-
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hybrid screen using the death domain of Fas as a bait (24). We showed that sentrin interacted with the signal–competent form of Fas and tumor necrosis factor receptor 1 and protected cells against anti-Fas or tumor necrosis factor-induced cell death (24). Sentrin is a novel protein with a ubiquitin homology domain (residues 22–97) that is 18% identical to ubiquitin (24). Four other laboratories have independently reported proteins with sequences identical to sentrin. These sequences were called UBL1 (ubiquitin-like 1) (25), PIFC1 (PML-interacting clone 1) (26), GMP1 (GAP modifying protein 1) (27), and SUMO-1 (small ubiquitin-related modifier) (28). We have shown that the COOH terminus of sentrin is efficiently cleaved to allow Gly to be used for conjugation to a subset of nuclear proteins (29). Furthermore, sentrin preferentially interacts with Ubc9 and forms a thiolester linkage with Ubc9 (30). In yeast, Ubc9 also forms a thiol ester conjugate with Ubc9 and forms a thiolester linkage with Ubc9 (30). In proteins (29). Furthermore, sentrin preferentially interacts with RanGAP1, PML, and Sp100. The amino-terminal part and RAR

Nickel Precipitation of RH-tagged PML Derivatives—To investigate the conjugation of sentrin to PML, PML-RH was expressed alone or co-expressed with HA-sentrin-1 in COS-M6 cells. Since the sequence of the COOH-tag is RGSHHHHH, the PML-RH derivatives can be purified by nickel-charged beads as described previously (21). Total cell lysate of the transfectants expressing PML-RH with or without HA-sentrin was prepared in the lysis buffer (6 mM guanidine hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride (pH 7.5)). DNA in the sample was sheared with a 22-gauge needle, and the lysate was centrifuged at 100,000 × g at 15 °C for 30 min. The supernatant was incubated with nickel-charged agarose resin beads (Invitrogen) for 1 h at room temperature. The beads were washed twice with the washing buffer (8 mM urea, 20 mM sodium phosphate, 500 mM sodium chloride (pH 7.8)), followed by two more rounds of washing with another washing buffer (8 mM urea, 20 mM sodium phosphate, 500 mM sodium chloride (pH 6.0)). Subsequently, the beads were washed with PBS twice and treated in 2% SDS treating solution for SDS-polyacrylamide gel electrophoresis analysis. The eluted proteins were analyzed by Western blotting using rabbit anti-sentrin antiserum or anti-HA monoclonal antibody (16B12) to detect PML-RH conjugated with native sentrin or HA-sentrin, respectively. To document expression level of PML-RH, anti-RH antibody was also used to detect unmodified PML-RH and its derivatives.

RESULTS

Sentrinization of the RING Finger Domain of PML—We have previously shown that wild type PML could be modified by sentrin, whereas PML-RARα-A and PML-RARα-B could not (21). Differential sentrinization of PML versus PML-RARα could play an important role in the pathogenesis of acute promyelocytic leukemia. Thus, it is of interest to define the sites in PML that are modified by sentrin or regulate the modification process. For this purpose, wild type and mutant PML molecules (see Fig. 1) were tagged with an RH epitope (RGSHHHHHH) at the COOH terminus and expressed in COS cells, as described previously (21, 35). Transfected cells were lysed with 10 mM guanidine HCl to denature proteins in the lysate and to prevent proteolysis or desentrinization. RH-tagged PML (PML-RH) was precipitated with nickel-charged beads and immunoblotted with either anti-sentrin-1 or anti-RH antibody to detect modified sentrin and total PML, respectively (21). Differential sentrinization of PML

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EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—COS-M6 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies—16B12 (Babco, Richmond, CA) is mouse monoclonal antibody to the peptide sequence YPYDVPDYA of influenza hemagglutinin (HA). Mouse anti-RH (specific for the amino acid sequence, RGSHHHHHH) monoclonal antibody was purchased from Qiagen (Santa Clara, CA). Rabbit polyclonal anti-sentrin antiserum was generated by the immunization with a peptide corresponding to the NH2-terminal 21 amino acids (29).

Plasmid Construction and Transfection—To express HA-tagged sentrin-1 in COS cells, pcDNA3/HA-sentrin-1 was transfected as described previously (29). The full-length cDNA of PML was prepared from the plasmid pMAMneoPML (33) and ligated into pcDNA3/RH-C (21) to generate pcDNA3/PML-RH. The cDNAs of PML mutants were made by polymerase chain reaction using appropriate primers, followed by the ligations with the vector, pcDNA3/RH-C. The full-length cDNA of RARα (34) was amplified by polymerase chain reaction from human testis cDNA library (Life Technologies, Inc.) as described previously (21, 35). The cDNA of PML-RARα-A was made by using primers pml-rar-a-F (5′-TGCGGC-GCGGAGGAGGACCCATTGAGACCCACCCACCA-3′) and pml-rar-a-R (5′-TGCTTGCTGCTGCTCATTGCTCCCGCCGAC-3′) complementary to pml-rar-a-F. The cDNA of PML-RARα-B was made by using primers pml-rar-b-F (5′-CATCACCCAGGGGAAAGCATTGGAGACCC-AGAGCA-3′) and pml-rar-b-R (5′-TGCTCTGCTGCTCATTGCTCCCGCCGAC-3′) complementary to pml-rar-b-F. The sequences of the inserts were confirmed by DNA sequencing analysis. To express RH-tagged PML, PML mutants, RARα, or PML-RARα, the plasmids described above were transfected into COS cells using LipofectAMINE (Life Technologies, Inc.) as described previously (21, 29, 35). The transfected cells were left untreated or were subjected to nickel precipitation or immunostaining 16 h after the incubation. For treatment with all-trans-RA (Aldrich), 16 h after transfection COS cell transfectants were incubated with 10−6 M RA for an additional 30 h and harvested.

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FIG. 1. Schematic of PML mutants (A) and PML-RARα fusion proteins (B).
Identification of Two Additional Sentrinization Sites—PML contains more than 20 lysine residues that are distributed throughout the entire molecule. We attempted to identify additional sentrinization sites by making lysine to arginine substitutions spanning the PML molecule. Analysis of a large number of substitution mutants led to the identification of Lys$^{65}$ as the second sentrinization site. This conclusion is supported by the experiments shown in Fig. 2B. As shown in lane 4, combining the K490R substitution with the K65R substitution abolished the upper two bands. Furthermore, addition of the K490R substitution to the RING finger domain led to loss of all three sentrinized bands (Fig. 2B, lane 6). Again, the mutant PML proteins were expressed at a level comparable with the PML proline-rich domain deletion mutant (lanes 7–12).

Since the PML(K65R,K490R) double mutant retained a single sentrinized band (Fig. 2B, lane 4), there must be an additional sentrinization site. The third sentrinization site was mapped to the B1 box, because combining the K65R substitution with the B1 box deletion led to loss of the upper two bands (Fig. 3A, lane 1). Complete loss of the three sentrinized bands was observed when the K490R substitution was added to the PML (K65R, B1 box deletion) mutant (Fig. 3A, lane 2). The B1 box contains three lysines at residues 133, 150, and 160. Thus, a Lys to Arg substitution was made to each residue and superimposed on the background of the PML(K65R,K490R) mutant. As shown in Fig. 3A, PML (K65R,K133R,K490R) (lane 4) and PML (K65R,K150R,K490R) (lane 5) still retained the lower band. However, PML (K65R,K160R,K490R) lost all three sentrinized bands. Again, the mutant proteins were expressed at a comparable level (lanes 7–12).

Interestingly, the PML(K160R) substitution mutant lost both the upper and middle bands, suggesting that this substitution affects Lys$^{160}$ and another site (Lys$^{65}$) (Fig. 3B, lane 3). This was supported by the observation that the K160R and the K65R,K160R mutants had an identical pattern of sentrinization, i.e. retaining only the lower band (Fig. 3D, lanes 3 and 4).

FIG. 2. Lys$^{65}$ in the RING finger domain is a major sentrinization site. A, COS cells expressing HA-tagged PML (lanes 1 and 7), RH-tagged PML (lanes 2 and 8), proline-rich domain deletion mutant (lanes 3 and 9), proline-rich domain and RING finger domain deletion mutant (lanes 4 and 10), nuclear localization signal deletion mutant (lanes 5 and 11), and COOH-terminal serine-proline-rich domain deletion mutant (lanes 6 and 12) were precipitated with nickel-charged beads and analyzed by Western blotting using rabbit anti-senstrin-1 antiserum (lanes 1–6) or mouse anti-RH monoclonal antibody (lanes 7–12). B, COS cells expressing RH-tagged proline-rich domain deletion mutant (lanes 1 and 7), proline-rich domain deletion plus K65R substitution (lanes 2 and 8), proline-rich domain deletion and K65R substitution (lanes 3 and 9), proline-rich domain deletion plus K65R and K490R substitutions (lanes 4 and 10), proline-rich domain and RING finger domain deletion (lanes 5 and 11), proline-rich domain and RING finger domain deletion plus K490R substitution (lanes 6 and 12) were precipitated with nickel-charged beads and analyzed by Western blotting using rabbit anti-senstrin-1 antiserum (lanes 1–6) or mouse anti-RH monoclonal antibody (lanes 7–12). Molecular size markers are shown in kilodaltons. C, PML mutants were co-expressed with HA-tagged sentrin-1, precipitated with nickel-charged beads, and analyzed by Western blotting using anti-HA monoclonal antibody (lanes 1–3) or upper two bands disappeared upon deletion of the both the proline-rich domain and the RING finger domain (lane 4). To ensure that the mutant PML proteins were expressed at a level comparable with the wild type PML, anti-RH mAb was used to detect the unmodified form of PML. As shown in lanes 8–12, the unmodified forms of PML were expressed equally well in all lanes.

These results suggest that the RING finger domain could contribute at least one sentrinization site. Interestingly, the RING finger domain of PML contains two lysine residues (Lys$^{65}$ and Lys$^{160}$) that may function as acceptors for sentrin. To investigate this possibility, RH-tagged PML(K65R) or PML(K68R) was expressed in COS cells and analyzed as described previously (21, 35). As shown in Fig. 2B, only the middle and lower bands were detected in the PML(K65R) sample (lane 2), whereas PML(K68R) (lane 3) retained all three bands. The faint upper band in lane 2 is most likely nonspecific, because it was not present in a separate experiment where HA-tagged sentrin was co-expressed with PML(K65R) (Fig. 2C, lane 2). These results suggest that the RING finger domain of PML contains one sentrinization site (Lys$^{65}$). Since deletion of the RING finger domain led to disappearance of the upper two bands (Fig. 2A, lane 4), the RING finger domain may also regulate sentrinization at a distant site.

Identification of Two Additional Sentrinization Sites—PML contains more than 20 lysine residues that are distributed throughout the entire molecule. We attempted to identify additional sentrinization sites by making lysine to arginine substitutions spanning the PML molecule. Analysis of a large number of substitution mutants led to the identification of Lys$^{65}$ as the second sentrinization site. This conclusion is supported by the experiments shown in Fig. 2B. As shown in lane 4, combining the K490R substitution with the K65R substitution abolished the upper two bands. Furthermore, addition of the K490R substitution to the RING finger domain led to loss of all three sentrinized bands (Fig. 2B, lane 6). Again, the mutant PML proteins were expressed at a level comparable with the PML proline-rich domain deletion mutant (lanes 7–12).

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Fig. 3. Identification of Lys<sup>65</sup> and Lys<sup>490</sup> as major sentrinization sites. A, COS cells expressing RH-tagged proline-rich domain and B1 box deletion plus K65R substitution (lanes 1 and 7), proline-rich domain and B1 box deletion plus K65,490R substitutions (lanes 2 and 8), proline-rich domain deletion plus K65R, K490R substitutions (lanes 3 and 9), proline-rich domain deletion plus K65R, K135R, K490R substitutions (lanes 4 and 10), proline-rich domain deletion plus K65R, K150R, K490R substitutions (lanes 5 and 11), proline-rich domain deletion plus K65R, K160R, K490R substitutions (lanes 6 and 12) were lysed, precipitated with nickel-charged beads, and analyzed by Western blotting using rabbit anti-sentrin-1 antiserum (lanes 1–6) or mouse anti-RH monoclonal antibody (lanes 7–12). Molecular size markers are shown in kilodaltons. B, RH-tagged PML mutants were co-expressed with HA-tagged sentrin-1-G, which could not be used in conjugation (lanes 1 and 7) or with HA-tagged sentrin-1 (all other lanes), precipitated with nickel-charged beads, and analyzed by Western blotting using anti-HA monoclonal antibody (lanes 1–6) or anti-RH monoclonal antibody (lanes 3 and 9), proline-rich domain deletion; lanes 4 and 10, proline-rich domain and RING finger domain deletion; lanes 5 and 11, nls deletion; lanes 6 and 12, COOH-terminal serine-proline-rich domain deletion. Molecular size markers are shown in kilodaltons.

Furthermore, the K160R, K490R mutant lost all sentrinization bands, similar to the K65R, K160R, K490R mutant (Fig. 3B, lanes 5 and 6). These results suggest that the RING domain and the B1 box could affect each other.

PML Mutant without the Nuclear Localization Signal Cannot Be Sentrinized—PML has a putative nuclear localization signal located between residues 476 and 490 (5, 37). Deletion of the nuclear localization signal (nls) led to the localization of PML in the cytosol (Ref. 37 and data not shown). As shown in Fig. 2A, lane 5, PML(Δnls) could not be sentrinized. Since Lys<sup>130</sup> is one of the sentrinized residues, we expected to observe at least two sentrinized bands (Lys<sup>65</sup> and Lys<sup>490</sup>) in PML(Δnls). The failure to sentrinize PML(Δnls) suggests, but does not prove, that PML may be normally sentrinized at either the nuclear pore or in the nucleus. Further experiments are required to demonstrate this point conclusively. Interestingly, PML(Δnls) could be sentrinized by overexpressing sentrin. This experiment was carried out by co-expressing RH-tagged PML proteins with HA-tagged sentrin in COS cells as described previously (21). As shown in Fig. 4, lane 5, a strongly positive sentrinized lower band and a faint upper band were observed. This pattern of sentrinization is not expected, because PML(Δnls) still contains two additional sentrinization sites (Lys<sup>65</sup> and Lys<sup>490</sup>) and was expected to show both the middle and lower bands. Thus, sentrinization of PML(Δnls) at the cytosol alters the normal sentrinization pattern of PML (see PML-RAI<sub>α</sub> below and “Discussion”). Overexpression of sentrin did not alter the sentrinization pattern of wild type PML (lane 2), proline-rich domain deletion mutant (lane 3), RING finger deletion mutant (lane 4), or the COOH-terminal serine, proline-rich domain deletion mutants (lane 6), however. Thus, when PML is localized in the nuclear compartment, overexpression of sentrin does not alter the sentrinization process.

Formation of Nuclear Body Is Dependent on Sentrinization in the Context of the RING Finger Domain and the B1 Box—The ability to form nuclear body or PML oncogenic domains is characteristic of wild type PML (10, 12). To assess the biological significance of PML sentrinization, we determined whether the PML sentrinization mutants described above could participate in nuclear body formation. As shown in Fig. 5A, wild type PML expressed in COS cells forms nuclear bodies as expected. PML with deletion of the NH<sub>2</sub>-terminal proline-rich domain or the COOH-terminal proline-serine rich domain could be sentrinized normally (Fig. 2A, lanes 3 and 6) and could form nuclear bodies similar to wild type PML (data not shown). PML(K65R), which lacks one sentrinization site (Fig. 2B, lane 2), could also form nuclear bodies similar to wild type PML (Fig. 5B). PML(K65R, K490R), which lacks two sentrinization sites (Fig. 3A, lane 3), had no obvious defect in nuclear body formation (Fig. 5C). The triple mutant PML(K65R, K160R, K490R) (Fig. 3, lane 6) showed poor nuclear body formation with homogenous nuclear staining (Fig. 5D). Deletion of the RING finger led to the loss of two sentrinization bands (Fig. 2A, lane 4) and complete loss of nuclear body formation (Fig. 5E). A B1 box deletion in the...
background of K65R retained a single sentrinized site (Lys490) (Fig. 3A, lane 1), but it did not show nuclear body formation (Fig. 5F). Thus, sentrinization in the context of the RING finger domain and the B1 box plays an important role in nuclear body formation. Sentrinization of Lys490 appears not to be essential for nuclear body formation.

Sentrinization of PML-RARα-A and PML-RARα-B Could Be Restored by Overexpression of Sentrin, but Not by RA Treatment—We have shown previously that PML-RARα-A and -B could not be modified by sentrin (21). Examination of the amino acid sequence of PML-RARα-A shows that it retains all three sentrinization sites, whereas PML-RARα-B contains two sentrinization sites with RING finger domain and the B1 box intact (see Fig. 1B). Thus, the inability to sentrinize PML-RARα fusion proteins was not due to a lack of sentrinization sites but to additional factors. One potential factor is cellular localization. This is particularly important for PML-RARα-B, because it is localized in the cytosol (Ref. 5 and data not shown). Since overexpression of sentrin could partly restore the sentrinization defect of PML(Δnls) (Fig. 4, lane 5), we asked whether PML-RARα-B could also be sentrinized when sentrin is overexpressed. As shown in Fig. 6A, lane 4, overexpression of sentrin also allowed PML-RARα-B to be sentrinized. As a control, RARα was not sentrinized with overexpression of sentrin (Fig. 6A, lane 2). PML-RARα-A is localized in both the nucleus and the cytosol (Ref. 5 and data not shown). PML-RARα-A is not normally sentrinized (21), but could also be sentrinized with overexpression of sentrin (Fig. 6A, lane 3). Since all-trans-retinoic acid could induce differentiation of APL cells, we asked whether retinoic acid could restore sentrinization of the PML-RARα fusion proteins. As shown in Fig. 6B, retinoic acid treatment did not appreciably restore sentrinization of PML-RARα-A and PML-RARα-B.

**DISCUSSION**

**PML Versus RanGAP1 Sentrinization**—PML is the second protein, after RanGAP1, shown to be covalently modified by sentrin (21–23). RanGAP1 is a 70-kDa protein consisting of leucine-rich repeats, an acidic domain, an α-helical domain, and a COOH-terminal domain (38). RanGAP1 is modified by a single sentrin molecule, because the modified RanGAP1 is 20 kDa larger than the unmodified form (21, 27, 28, 35). The unmodified RanGAP1 is localized to the cytosol, whereas the sentrinized RanGAP1 is localized to the nuclear pore complex. Recently, Lys526 of RanGAP1 has been shown to be the sentrin modification site (39, 40). In contrast to RanGAP1, PML consists of an NH2-terminal proline-rich domain, followed by a RING finger domain, two zinc finger-like B boxes, an α-helical domain, a nuclear localization signal, and a COOH-terminal proline-serine-rich domain. Comparison of the sentrinization
sites of PML with RanGAP1 does not yield any useful clues about a general sentrinization motif.

In COS cells overexpressing PML, about 30% of the PML existed in the sentrinized form (this report). Similar observations have also been made in stable transfected cell lines (21–23). Thus, there appears to be a dynamic equilibrium in PML sentrinization. The unmodified PML migrates at approximately 75 kDa, whereas the lowest sentrinized band migrates at approximately 66 kDa. Thus, it appears that PML-RARα fusion protein in the myeloid- promyelocytic lineage (43). Several laboratories have postulated that PML-RARα functions as a dominant negative oncogene in disrupting nuclear body formation and in inhibiting normal PML activity (5, 10, 12). Sentrinization of PML clearly plays an important role in the formation of nuclear body. The failure of PML-RARα to form nuclear bodies could be attributed to its inability to be sentrinized. Since RA treatment could induce differentiation of APL cell lines and clinical remission in APL patients, we initially expected RA to induce sentrinization of PML-RARα. The inability to induce sentrinization of PML-RARα suggests that RA has a mode of action that is not mediated by sentrinization. This is consistent with a recent report that RA could induce degradation of PML-RARα (22, 44). It is intriguing that overexpression of sentrin could induce sentrinization of PML-RARα. At present, it is not known whether sentrinization of PML-RARα would alter its biological activity. The mutational analyses presented in this report should provide the basis for further examination of the structure/function relationship of PML and PML-RARα and in a better understanding of the pathogenesis of APL.

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