PML-RARα and Its Phosphorylation Regulate PML Oligomerization and HIPK2 Stability

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Abstract

The PML gene is frequently fused to the retinoic acid receptor α (RARα) gene in acute promyelocytic leukemia (APL), generating a characteristic PML-RARα oncogenic chimera. PML-RARα disrupts the discrete nuclear speckles termed nuclear bodies, which are formed in PML, suggesting that nuclear body disruption is involved in leukemogenesis. Nuclear body formation that relies upon PML oligomerization and its stabilization of the hypoxia-inducible protein kinase (HIPK)-2 is disrupted by expression of the PML-RARα chimera. Here, we report that disruption of nuclear bodies is also mediated by PML-RARα inhibition of PML oligomerization. PKA-mediated phosphorylation of PML-RARα blocked its ability to inhibit PML oligomerization and destabilize HIPK2. Our results establish that both PML oligomerization and HIPK2 stabilization at nuclear bodies are important for APL cell differentiation, offering insights into the basis for the most common prodifferentiation therapies of APL used clinically. Cancer Res; 73(14): 4278–88. ©2013 AACR.

Introduction

In human leukemia, specific chromosomal translocations result in the expression of fusion proteins promoting malignancy (1, 2). The PML gene is the target of the t(15;17) chromosome translocation that is observed in more than 90% of acute promyelocytic leukemia (APL) cases, in which fusion of the PML and retinoic acid receptor α (RARα) genes leads to the expression of the aberrant PML-RARα fusion protein (3–6). The PML protein normally forms discrete nuclear speckles in the nucleus called PML nuclear bodies; in these, PML recruits other proteins, including transcription factors such as p53 and acute myeloid leukemia 1 (7–9), transcription coactivators such as hypoxia-inducible protein kinase (HIPK)-2 and p300 (10, 11), SUMO (12), and DAXX (13, 14). Nuclear bodies have been implicated in the regulation of apoptosis, cellular senescence, and antiviral responses. It has been reported that PML stabilizes transcription coactivators, such as HIPK2 and p300, to assemble transcription factor/coactivator complexes within nuclear bodies (15). In contrast, nuclear bodies are disrupted in t(15;17) APL (12, 16–19); in the presence of the PML-RARα fusion protein, nuclear bodies appear as dispersed microspeckles in which HIPK2 is destabilized (15). All-trans-retinoic acid (ATRA) and As2O3, which are used clinically in APL, restore the normal appearance of nuclear bodies (12, 17, 19–21). In t (15;17) APL, it remains unclear that the disruption of nuclear bodies is related to leukemogenesis and that their restoration would lead to therapy. The molecular mechanism by which PML-RARα disrupts nuclear bodies has remained elusive, hampering progress in the understanding of leukemogenesis.

The present study reveals that PML-RARα blocks PML oligomerization and disrupts nuclear bodies, and that the effect is reversed upon cAMP/PKA-dependent phosphorylation of PML-RARα. In addition, pharmacologic activation of adenylyl cyclase by forskolin restores PML nuclear bodies and promotes ATRA-induced APL cell differentiation. Furthermore, nuclear body restoration induced HIPK2 stabilization. These results suggest that nuclear body formation is regulated by PML-RARα phosphorylation, and that the restoration of nuclear bodies is important for APL cell differentiation.

Materials and Methods

Cell culture, infection, and antibodies

293FT cells, which were purchased from Invitrogen, and U2OS cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Plat-E cells were obtained from Dr. T. Kitamura (University of Tokyo, Tokyo, Japan) and were cultured in DMEM supplemented with 10% FCS, 10 μg/mL blasticidin, and 1 μg/mL puromycin. NB4 cells and K562 cells were cultured in RPMI-1640 medium supplemented with 10% FCS. Anti-HIPK2 antibody was described previously (15). Other antibodies were purchased commercially: anti-HA (3F10, Roche and Y11, Santa Cruz), anti-FLAG (M2, Sigma), anti-Myc (9E10, Upstate), anti-tubulin (H235, Santa Cruz), anti-MLL (16.1–104, Upstate; H238, Santa Cruz; 001, MBL), anti-SUMO (Zymed), anti-DAXX (Exbio), and anti-RARα (C20, Santa Cruz).
Plasmids
The PML, PML-RAR\(\alpha\), and HIPK2 expression vectors were generated as described previously (15). PML and PML-RAR\(\alpha\) deletion mutants were generated by PCR using pLNCX-HA-PML IV or pLNCX-HA-PML-RAR\(\alpha\) as the template.

Immunoprecipitation and Western blotting
293FT cells were transfected with the desired vectors and lysed as described previously (15). Cell lysates and immunoprecipitates were fractionated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amerham). The membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were visualized by the enhanced chemiluminescence (ECL) or ECL-Plus technique (Amersham), and the images were analyzed by ImageGauge (FUJIFILM).

Immunofluorescence
U2OS cells were cultured in 4-well chamber slides and transfected as described previously (15). The cells were exposed to 50 \(\mu\)M forskolin for 18 hours. After forskolin exposure, the cells were fixed and incubated with antibodies as described previously. PML-RAR\(\alpha\)-expressing mouse c-kit\(^+\) cells were exposed to 50 \(\mu\)M forskolin for 24 hours. NB4 cells were exposed to 50 \(\mu\)M forskolin or 1 \(\mu\)M ATRA for 24 or 72 hours. The cells were analyzed by the same techniques as U2OS cells. Images were captured on an Olympus microscope and analyzed by deconvolution.

Serial replating assay
pMSCV, pMSCV-HA-PML-RAR\(\alpha\), pMSCV-HA-PML-RAR\(\alpha\) \(\Delta E\), pMSCV-HA-PML-RAR\(\alpha\) S704A, pMSCV-HA-PML-RAR\(\alpha\) S704D, and pMSCV-HA-HIPK2 were transfected into Plat-E cells using Genejuice (Novagen), and supernatants containing retrovirus were collected 48 hours after transfection. C-kit\(^+\) cells were selected from the femurs of C57BL/6 mice using CD117-specific MicroBeads (Miltenyi Biotech), transduced with retroviruses using RetroNectin (Takara), and plated in methylcellulose medium (M3434, StemCell Technologies). The cells were cultured and replated every 4 to 6 days in methylcellulose medium under G418 selection. The cells from the first round (empty vector and PML-RAR\(\alpha\) \(\Delta E\)) or the third round (PML-RAR\(\alpha\) point mutants) of colonies were harvested and analyzed by using immunofluorescence as described above. With respect to HIPK2 stabilization, C-kit\(^+\) cells were infected with retroviruses containing HA-HIPK2. The next day, the cells were infected with retroviruses containing wild-type or mutants of HA-PML-RAR\(\alpha\). The cells were cultured in methylcellulose medium under G418 and puromycin selection. The cells of the third-round colonies were harvested and subjected to Western blot analysis.

RT-PCR
Reverse transcriptase PCR was conducted as described previously (15). HIPK2 mRNA expression was analyzed using the following primers: forward (5’-CCCTCCAAATACTTT-CGCC-3’) and reverse (5’-TGGTGTCCTTCAGTCCACA-AAGG-3’). The glyceraldehyde-3-phosphate dehydrogenase primer set was described previously (15).

Flow cytometric analysis
Before incubation with anti-Mac1-FITC (M1/70, eBioscience), NB4 cells were preincubated with immunoglobulin G from rat serum (Sigma) to prevent nonspecific binding of the antibody. The stained cells were analyzed by JSAN (Bay Bioscience), and the results were analyzed using FLOWJO software.

Results
PML oligomerization is required for nuclear body formation
The coiled-coil domain of PML mediates homo-oligomerization and hetero-oligomerization (18, 22–24). We have previously shown that PML IV interacts with 2 transcription factors important for granulocytic differentiation, PU.1 and CAAT/enhancer-binding protein (C/EBP\(e\); refs. 25, 26). Therefore, we used PML IV and a PML mutant lacking the coiled-coil domain (PML IV \(\Delta C\); Fig. 1A). As expected, PML IV \(\Delta C\) could not form homo-oligomers (Supplementary Fig. S1A). Immunofluorescence was conducted to assess the localization of the PML IV \(\Delta C\) mutant. As shown previously (21, 27), wild-type PML IV localized to the nucleus and formed nuclear bodies of normal appearance; however, the PML IV \(\Delta C\) mutant was expressed uniformly throughout the nucleus and did not form nuclear bodies (Supplementary Fig. S1B). These results suggest that PML oligomerization is associated with nuclear body formation. HIPK2 is stabilized by PML in nuclear bodies and destabilized by PML-RAR\(\alpha\) (15). To test whether the PML mutant destabilized HIPK2, FLAG-tagged HIPK2 was cotransfected with HA-tagged PML IV or PML IV \(\Delta C\). Results showed that PML IV stabilized HIPK2, which is consistent with previous observations (15); however, PML IV \(\Delta C\) destabilized HIPK2 (Fig. 1B). The destabilization was rescued by a proteasome inhibitor, MG132 (Fig. 1B). These results suggest that PML oligomerization is required for PML-mediated HIPK2 stabilization.

PML-RAR\(\alpha\) disrupts nuclear bodies; however, the molecular mechanism underlying this effect remains unclear. The defect in nuclear body formation by the oligomerization-deficient PML IV \(\Delta C\) mutant led to the hypothesis that PML-RAR\(\alpha\) may prevent PML oligomerization. To test this hypothesis, the effect of PML-RAR\(\alpha\) on nuclear body disruption was evaluated by immunofluorescence. Wild-type PML and PML-RAR\(\alpha\) are expressed in APL cells. The localization of PML is diffused in cells expressing PML-RAR\(\alpha\) (18). We transfected U2OS cells with expression vectors encoding FLAG-tagged PML IV and hemagglutinin (HA)-tagged PML IV or HA-tagged PML-RAR\(\alpha\). PML nuclear bodies were detected using anti-FLAG antibody. As shown in Supplementary Fig. S2B, PML IV formed large, discrete, and distinct nuclear foci in the absence of PML-RAR\(\alpha\) (top) but small and disperse foci in the presence of PML-RAR\(\alpha\) (bottom). The oligomerization capacity of PML was subsequently assessed in the presence and absence of PML-RAR\(\alpha\). As shown in Supplementary Fig. S2C, PML-RAR\(\alpha\) inhibited PML–PML interaction, suggesting
domain of PML-RAR

![Figure 1](image-url)

Figure 1. The coiled-coil domain of PML is required for HIPK2 stabilization. A, diagram of the PML deletion mutant. The proline-rich region (PrO), the ring-finger domain (RfG), the coiled-coil domain (CC), the nuclear-import signal (NLS), and the serine-proline-rich region (SP) are indicated. B, PML IV ΔCC destabilizes HIPK2. 293FT cells were transfected with pLNCX-FLAG-HIPK2 and empty vector, pLPCX-HA-PML IV, or pLPCX-HA-ΔCC. The cells were treated with or without 10 μmol/L MG132 for 16 hours. The expression of HIPK2 (top), PML IV, or PML IV ΔCC (middle) and tubulin (bottom) was detected by immunoblotting using anti-FLAG, anti-HA and anti-tubulin antibodies, respectively.

that PML-RARα disrupts PML nuclear bodies by inhibiting PML oligomerization. The N-terminal or the coiled-coil domain of PML-RARα has been reported to be important for nuclear body disruption (28). PML 1–394, which is the PML moiety of PML-RARα and lacks the PML C-terminal, did not disrupt PML nuclear bodies (Supplementary Fig. S2D), suggesting that the RARα moiety of the fusion protein is also important for nuclear body disruption.

The ligand-binding domain of the RARα moiety of PML-RARα is essential for nuclear body disruption

Because the RARα moiety of PML-RARα is important for the disruption of PML nuclear bodies, PML-RARα deletion mutants were generated (Fig. 2A) to identify the region of RARα required for the effect. HA-tagged deletion mutants were cotransfected with FLAG-tagged PML IV. Immunofluorescence analysis indicated that PML-RARα 1–708 or 1–661 was cotransfected with FLAG-tagged PML IV. PML-RARα 1–708 led to the formation of microspeckle PML nuclear bodies, whereas PML-RARα 1–661 did not (Supplementary Fig. S3B). These results indicate that the PML-RARα region spanning amino acids 662 to 708 is required for PML nuclear body inhibition. RARα and PML-RARα are phosphorylated by PKA (29, 30). Because serine 704, the PKA-dependent phosphorylation site of PML-RARα, is located within the region required for nuclear body disruption (Fig. 3A), mutants were generated in which serine 704 was substituted by alanine and aspartate to simulate dephosphorylated and phosphorylated serine, respectively. The effect of these mutants on PML nuclear body formation was assessed by the transfection of U2OS cells. Representative immunofluorescence data are shown in Fig. 3B (left), and the expression levels of PML and PML-RARα are shown in Fig. 3C. Quantification was done by counting the number of cells that formed large, discrete, and distinct PML nuclear foci in all transfected cells (Fig. 3D). Interestingly, wild-type PML-RARα and the alanine mutant (S704A) disrupted nuclear bodies, as shown by the presence of microspeckles, whereas the aspartate mutant (S704D) did not. Both mutants interacted with PML IV as strongly as did wild-type PML-RARα and the ΔE mutant (Supplementary Fig. S4), suggesting that the inability to disrupt nuclear bodies is not due to any deficiency in binding to PML.

Forskolin was then used to activate adenyl cyclase to
determine whether cAMP/PKA might restore nuclear bodies by phosphorylating the serine residue of PML-RARα. In the presence of forskolin, wild-type PML-RARα did not affect nuclear bodies (Fig. 3B and D); however, the S704A mutant, which lacks the serine residue phosphorylated by PKA, inhibited PML nuclear body formation even in the presence of forskolin.

We also tested the effect of wild-type and mutant PML-RARα in normal myeloid stem/progenitor cells. C-kit+ mouse myeloid stem/progenitor cells were infected with a retrovirus encoding HA-tagged PML-RARα, HA-tagged S704A, or HA-tagged S704D and cultured in methylcellulose medium. The location of PML in the immortalized cells was assessed using an anti-PML antibody specific for mouse Pml. Representative immunofluorescence data are shown in Fig. 4A, and quantification is shown in Fig. 4B. Nuclear body formation was maintained in S704D-expressing cells but was largely disrupted in wild-type PML-RARα- and S704A-expressing cells. The cells transduced with S704D were immortalized as well as those transduced with PML-RARα and S704A (Fig. 4C). Wild-type PML-RARα, S704A, and S704D were expressed at similar levels in the immortalized cells (Fig. 4D). Forskolin restored PML nuclear bodies in the wild-type PML-RARα-expressing cells but could hardly restore nuclear bodies in the S704A-expressing cells (Fig. 4E). These results suggest that the ability of PML-RARα to disrupt nuclear bodies is inhibited by cAMP/PKA-mediated phosphorylation of PML-RARα at serine 704.

Disruption of PML nuclear bodies by PML-RARα is strongly correlated with the destabilization of HIPK2 and the inhibition of PML oligomerization by PML-RARα

As shown in Fig. 1B and Supplementary Fig. S1B, PML IV ΔCC did not form nuclear bodies and destabilized HIPK2. Similarly, the PML-RARα deletion mutants that inhibited PML nuclear body formation also destabilized HIPK2 (Fig. 2F). These results suggest a correlation between nuclear body formation and HIPK2 stability. Therefore, the PML-RARα point mutants described above were assessed for their effect on HIPK2 stability. As shown in Fig. 5A, the level of HIPK2 decreased when HIPK2 was cotransfected with wild-type PML-RARα or S704A but not when HIPK2 was cotransfected with S704D. The effect of the point mutants on HIPK2 stability was also assessed in a stable system as shown in Fig. 4. Endogenous mouse HIPK2 could not be detected (data not shown). The expression of HA-tagged human HIPK2, introduced in mouse c-kit+ cells, was only detected in S704D-expressing cells (Fig. 5B) of the third-round colonies. However, HIPK2 mRNA levels in cells expressing wild-type PML-RARα, S704A, or S704D were not appreciably different (Supplementary Fig. S5). These data support the hypothesis that HIPK2 destabilization is associated with PML nuclear body disruption. The point mutants were then assessed for their effect on PML oligomerization. S704A inhibited PML homo-oligomerization, as did wild-type PML-RARα, whereas S704D did not (Fig. 5C), implying that the protection of PML oligomerization from PML-RARα promotes PML nuclear body formation.

PKA-dependent phosphorylation of PML-RARα restores nuclear bodies and promotes ATRA-induced APL cell differentiation

As shown in Figs. 3–5, PKA-dependent phosphorylation of PML-RARα may be the switch that restores nuclear bodies. Cyclic AMP (cAMP) alone has no effect on nuclear body restoration, but cAMP and ATRA cooperatively restore nuclear bodies in NB4-R1 cells, which are ATRA-maturation-resistant cell lines (31). These data suggest that the cAMP/PKA pathway plays an important role in nuclear body formation. To determine whether the cAMP/PKA pathway actually regulates nuclear body formation in APL cells, APL-derived NB4 cells, which express endogenous PML-RARα, were exposed to forskolin alone. Nuclear bodies were detected using an anti-PML antibody; nuclear bodies were disrupted in NB4 cells, and became clear in the presence of forskolin (Supplementary Fig. S6A). Forskolin did not induce NB4 cell differentiation (Supplementary Fig. S6A). To characterize the forskolin-induced clear particles, the localization of SUMO and DAXX, which are recruited to nuclear bodies, was assessed using anti-SUMO and anti-DAXX antibodies. In the absence of forskolin or ATRA, the colocalization of SUMO and PML, or DAXX and PML, was very limited (Supplementary Fig. S6B). In contrast, ATRA restored nuclear bodies and recruited SUMO and DAXX to nuclear bodies. Forskolin changed the appearance of PML nuclear bodies from microspeckles to clear particles. In the clear particles, PML colocalized with SUMO and DAXX (Supplementary Fig. S6B). Thus, forskolin alone, like ATRA, restored nuclear bodies. ATRA induced PML-RARα degradation, whereas forskolin did not (Supplementary Fig. S6C). These data might reflect the smaller size of nuclear bodies in the presence of forskolin compared with that of ATRA. These data also suggest that PML-RARα phosphorylated by cAMP/PKA inhibits nuclear body disruption.

Because forskolin restored nuclear bodies in NB4 cells, the stability of endogenous HIPK2 was assessed in NB4 cells exposed to forskolin. As shown in Fig. 6A, forskolin increased HIPK2 protein levels but not HIPK2 mRNA levels. In contrast, forskolin did not increase HIPK2 protein levels in the non-APL K562 cells (Fig. 6B). HIPK2 protein levels increased in NB4 cells exposed to ATRA for 24 hours (Fig. 6C). Time-course analysis indicated that the increase in HIPK2 expression was correlated with nuclear body restoration (Fig. 6C and D). These results suggest that HIPK2 is stabilized in nuclear bodies restored upon cAMP/PKA-mediated phosphorylation of PML-RARα.

Finally, NB4 cells were exposed to forskolin or/and ATRA to determine whether nuclear body restoration may promote NB4 cell differentiation. As shown previously, forskolin was not sufficient to induce NB4 cell differentiation (Fig. 6E and F); however, forskolin enhanced ATRA-induced differentiation (Fig. 6E and F). The combination of ATRA and forskolin resulted in the differentiation of NB4 cells into segmented granulocytes (Fig. 6F) and increased the expression of the differentiation marker Mac-1 (Fig. 6G) more efficiently than either drug alone. Other studies have also shown the efficacy of cAMP against APL (32–35). These results and reports suggest that cAMP/PKA promotes ATRA-induced APL cell differentiation by restoring nuclear bodies.
A

B

C

D

E

F

G

ΔE

ΔC

PML-RARα deletion derivatives

PML IV

PML-RARα deletion derivatives

PML IV

PML IV

PML IV

PML IV

PML IV

PML IV
Figure 2. The ligand-binding domain of PML-RARα is essential for nuclear body disruption. A, diagram of PML-RARα deletion mutants. B, the ligand-binding domain of PML-RARα is required for the disruption of PML nuclear bodies. U2OS cells were transfected with pLNCX-FLAG-PML IV and pLNCX-HA-PML-RARα deletion constructs or only with pLNCX-HA-PML-RARα deletion constructs as described. PML nuclear bodies were analyzed using anti-FLAG antibody. The white bar represents 10 μm. C, expression of PML-RARα deletion mutants. The expression of PML IV (top), PML-RARα deletion mutants (middle), and tubulin (bottom) in U2OS cells was detected by immunoblotting using anti-FLAG, anti-HA, and anti-tubulin antibodies, respectively. D, quantification of nuclear body formation. The number of cells with PML nuclear bodies was counted. Values represent the mean ± SEM of 3 independent experiments.

Figure 3. The PKA-dependent phosphorylation site of PML-RARα regulates PML nuclear body formation. A, diagram of the location of the PKA-dependent phosphorylation site of PML-RARα. B, phosphorylation of serine 704 is important for the restoration of PML nuclear bodies. FLAG-tagged PML IV and HA-tagged PML-RARα point mutants were coexpressed in U2OS cells. Cells were exposed to 50 μmol/L forskolin. PML nuclear bodies were analyzed using an anti-FLAG antibody. The white bar represents 10 μm. C, expression of PML-RARα wild-type and point mutants. The expression of PML IV (top), PML-RARα wild-type or point mutants (middle), and tubulin (bottom) in U2OS cells was detected by immunoblotting using anti-Myc and anti-HA antibody. The values of IP/input intensity of Myc-PML IV were quantified using ImageGauge and normalized to the value of Myc-PML IV/empty vector/FLAG-PML IV. DAPI, 4',6-diamidino-2-phenylindole; NB, nuclear body.

Discussion

In more than 90% of APL cases, the PML-RARα fusion protein is generated by the [t(15;17)] chromosomal translocation. PML-RARα disrupts PML nuclear bodies by a mechanism that was not understood in detail. The present study reveals that PML-RARα blocks PML oligomerization, resulting in the disruption of nuclear bodies, and that cAMP/PKA phosphorylation of PML-RARα restores nuclear bodies. Our results suggest that nuclear body restoration enhances APL cell differentiation.
Figure 4. Nuclear body formation is maintained in cells stably expressing PML-RARα S704D. A, PML-RARα S704D does not inhibit nuclear body formation. C-kit⁺ mouse bone marrow cells were infected with pMSCV-HA-PML-RARα wild-type, S704A, or S704D. Endogenous murine PML nuclear bodies of cells at the third round of colonies were analyzed using mouse Pml-specific antibody (16.1–104). The white bar represents 10 μm. The thick arrow represents PML nuclear bodies, arrowhead represents intermediate nuclear bodies, and thin arrow represents microspeckles. B, quantification of nuclear body formation. The number of cells with PML nuclear bodies, intermediate nuclear bodies, and microspeckles was counted. Values represent the average of 4 independent experiments. C, the cells expressing PML-RARα S704D are immortalized. C-kit⁺ mouse bone marrow cells were infected with empty vector (mock), pMSCV-HA-PML-RARα wild-type, S704A, or S704D and cultured in methylcellulose medium. The colony number from the third to the fifth round of colonies is indicated (top). Values represent mean ± SEM from 3 independent experiments. The cells at the third round of colonies were stained with May–Giemsa stain (bottom). D, expression of PML-RARα wild-type, S704A, and S704D. The expression of wild-type PML-RARα and mutants and of tubulin in cells at the third round of colonies was analyzed by immunoblotting using anti-PML and anti-tubulin antibodies, respectively. E, quantification of nuclear body restoration. The cells expressing wild-type PML-RARα and S704A at the third round of colonies were collected and exposed to 50 μmol/L forskolin for 24 hours. Endogenous murine PML nuclear bodies were analyzed as described in A. The number of cells with PML nuclear bodies, intermediate nuclear bodies, or microspeckles was counted. Values represent the average of 4 independent experiments. DAPI, 4',6-diamidino-2-phenylindole; NB, nuclear body; WT, wild-type.

PML nuclear body disruption and restoration

Nuclear bodies are disrupted in APL cells harboring the t(15;17) chromosomal translocation (16, 17, 19). Results showed that wild-type PML-RARα blocked PML oligomerization (Supplementary Fig. S2C). Deletion analysis showed that PML-RARα mutants that block PML oligomerization...
interrupted in U2OS cells expressing wild-type PML-RAR bodies. Moreover, forskolin restored nuclear bodies by PML-RAR (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RAR point mutants on HIPK2 stability. FLAG-tagged HIPK2 was expressed with either empty vector or HA-tagged PML-RAR point mutants. The expression of HIPK2, PML-RAR (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RAR point mutants on HIPK2 stability.

293FT cells were transfected with pLNCX-Myc-PML IV and either pLNCX-HA-PML-RAR (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RAR point mutants on HIPK2 stability. FLAG-tagged HIPK2 was expressed with either empty vector or HA-tagged PML-RAR point mutants. The expression of HIPK2, PML-RAR (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RAR point mutants on HIPK2 stability.

Figure 5. HIPK2 destabilization and inhibition of PML oligomerization are correlated with PML nuclear body disruption by PML-RARα. A, effect of the PML-RARα point mutants on HIPK2 stability. FLAG-tagged HIPK2 was expressed with either empty vector or HA-tagged PML-RARα point mutants. The expression of HIPK2 (top), PML-RARα point mutants (middle), and tubulin (bottom) was detected by immunoblotting using anti-FLAG, anti-HA, and anti-tubulin antibodies, respectively. B, effect of the PML-RARα point mutants on HIPK2 stability in a stable expression system. C, Ito mouse bone marrow cells were infected with pMSCV-HA-HIPK2 and pMSCV-HA-PML-RARα encoding either wild-type, S704A, or S704D. The expression of HIPK2, PML-RARα (wild-type and mutants), and tubulin at the third round of colonies was analyzed by immunoblotting using anti-HIPK2, anti-PML, and anti-tubulin antibodies, respectively. C, effect of the PML-RARα point mutants on HIPK2 stability.

Also disrupt nuclear bodies (Figs. 2B and G, 3B, and 5C). The blocking of PML oligomerization by PML-RARα occurs independent of their interaction (Figs 2G and 5C). These results suggest that the inability of inhibition of PML oligomerization by PML-RARα mutants is not due to the inability of interaction with PML IV, and imply that PML-RARα-induced nuclear body disruption is due to impaired PML oligomerization.

RARα and PML-RARα are phosphorylated by the cAMP/PKA pathway at a site located within the ligand-binding domain (29, 30). The PML-RARα S704D mutant, which is expected to simulate phosphorylated PML-RARα, did not block PML oligomerization and did not disrupt nuclear bodies. Moreover, forskolin restored nuclear bodies disrupted in U2OS cells expressing wild-type PML-RARα (Fig. 3B and D), in mouse myeloid stem/progenitor cells expressing wild-type PML-RARα (Fig. 4E), or in APL-derived NB4 cells (Fig. 6D). Our results indicate that the ligand-binding domain of PML-RARα is key to the inhibition of PML oligomerization and to the disruption of PML nuclear bodies. Also disrupt nuclear bodies (Figs. 2B and G, 3B, and 5C). The blocking of PML oligomerization by PML-RARα occurs independent of their interaction (Figs 2G and 5C). These results suggest that the inability of inhibition of PML oligomerization by PML-RARα mutants is not due to the inability of interaction with PML IV, and imply that PML-RARα-induced nuclear body disruption is due to impaired PML oligomerization.

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**Nuclear body restoration is important for differentiation of APL cells**

The PKA phosphorylation site of PML-RARα regulates nuclear body formation. Forskolin restored nuclear bodies in
APL-derived NB4 cells (Fig. 6D). Although forskolin alone was not sufficient to induce NB4 cell differentiation, it promoted ATRA-induced differentiation (Fig. 6G). Published studies showed that cAMP enhances retinoic acid-induced APL differentiation and PML-RARα transactivation (32–35). These studies also showed that PKA dissociates RARα from SMRT and activates transcription. Moreover, a recent report indicated that cAMP-dependent phosphorylation of PML-RARα was crucial for the eradication of APL-initiating cells (30). Taken together, these reports and the present data suggest that nuclear body restoration is one of the reasons why cAMP/PKA could be useful as an APL therapy.

We previously showed that HIPK2 is stabilized in PML nuclear bodies and degraded outside of nuclear bodies by SCFFbx3, suggesting that HIPK2 is destabilized by disruption of nuclear bodies (15). In this paper, we showed that PML IVΔCC, wild-type PML-RARα, PML-RARα1–748, and S704A, which disrupted PML nuclear bodies, also destabilized HIPK2. In contrast, PML, PML-RARα1–567, 1–492, 1–420, ΔE, and S704D, which did not disrupt nuclear bodies.
did not destabilize HIPK2. Forskolin and ATRA, which restore nuclear bodies in NB4 cells, increased HIPK2 expression (Fig. 6A and C), and the increase in HIPK2 expression was correlated with nuclear body restoration (Fig. 6D). These data indicate that HIPK2 destabilization is strongly correlated with nuclear body disruption and that nuclear body formation is important for HIPK2 stabilization. HIPK2 is important for PML-dependent transcriptional activation (15). We have also found that PML stabilizes the PU.1/p300 complex to regulate PU.1-dependent transcription and myeloid differentiation (26). Mutations of HIPK2 are found in AML and myelodysplastic syndrome (36). PML-RARα also disrupts PU.1/p300 complexes and inhibits myeloid differentiation (26). Therefore, nuclear body formation by PML oligomerization may lead to the recruitment of transcription factors/co-activators and to their stabilization for transcriptional activation and regulation of granulopoiesis. As suggested by the results of the present study, this might be because cAMP/PKA-dependent nuclear body restoration enhances APL cell differentiation.

References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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PML-RARα and Its Phosphorylation Regulate PML Oligomerization and HIPK2 Stability

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