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PML-Retinoic Acid Receptor α Inhibits PML IV Enhancement of PU.1-Induced C/EBPε Expression in Myeloid Differentiation▽†

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PML and PU.1 play important roles in myeloid differentiation. PML-deficient mice have an impaired capacity for terminal maturation of their myeloid precursor cells. This finding has been explained, at least in part, by the lack of PML action to modulate retinoic acid-differentiating activities. In this study, we found that C/EBPε expression is reduced in PML-deficient mice. We showed that PU.1 directly activates the transcription of the *C/EBP*ε gene that is essential for granulocytic differentiation. The type IV isoform of PML interacted with PU.1, promoted its association with p300, and then enhanced PU.1-induced transcription and granulocytic differentiation. In contrast to PML IV, the leukemia-associated PML-retinoic acid receptor α fusion protein dissociated the PU.1/PML IV/p300 complex and inhibited PU.1-induced transcription. These results suggest a novel pathogenic mechanism of the PML-retinoic acid receptor α fusion protein in acute promyelocytic leukemia.

Acute promyelocytic leukemia (APL) has been characterized as a differentiation arrest at the promyelocyte stage due to t(15;17) reciprocal chromosomal translocation that generates a PML-retinoic acid (RA) receptor α (RARA) fusion protein (3, 10). All-*trans* RA (ATRA) induces the differentiation and elimination of APL clones (35). Biochemical evidence that RARA and PML-RARA are bidirectional transactivators (12) and the participation of the *RARA* gene in all APL syndromes examined so far (36) suggest that dominant negative inhibition of RA signaling by PML-RARA plays a critical role in the pathogenesis of APL (25). However, RARA-deficient ($RARA^{-/-}$) mouse models revealed that the retinoid signal is dispensable for myeloid differentiation (11); therefore, how PML-RARA leads to APL requires a revision.

PML contains a characteristic triad of a RING finger, two B boxes (B1 and B2), and a coiled-coil motif, which participates in the formation of high-order multiprotein complexes (20). PML forms discrete "speckle" structures in the nucleus called PML oncogenic domains (PODs) (40). Although the biological functions of PODs remain unclear, they are disrupted by PML-RARA into "microspeckle" structures, which are a hallmark of APL (20). There are several isoforms of PML, which differ in their C termini as a result of alternative splicing (9). Although PML proteins have a number of pleiotropic functions, such as regulation of proliferation, apoptosis, or senescence, at least in vitro (20), little is known about the specific activities of each isoform. PML has been highlighted as a transcriptional coregulator, since it associates with several transcription factors and cofactors (40). Despite the ubiquitous expression of PML proteins and the variety of binding partners, PML-deficient ($PML^{-/-}$) mice do not display significant phenotypes, suggesting that they are not required for but rather modulate normal development. Interestingly, however, the terminal differentiation of granuloid and monocytoid cell lineages is impaired in $PML^{-/-}$ mice (32), but how this occurs remains to be elucidated.

Granulopoiesis is a tightly regulated developmental process that begins with the commitment of myeloid precursors followed by their terminal differentiation, a process that requires cooperative or stepwise actions of lineage-specific transcription factors (6). PU.1 is expressed exclusively in hematopoietic cells (13), and it binds to a purine-rich DNA sequence containing the 5′-GGAA/T-3′ core motif. Although the targeted disruption of the *PU.1* gene can cause multiple hematopoietic aberrations, it invariably causes a defect in the terminal differentiation of myeloid cells (5, 19, 22, 27). PU.1 associates with the p300/CREB-binding protein (CBP) coactivator, at least in vitro (33); however, its essential protein-protein interactions during granulocytic terminal differentiation remain to be explored. PU.1 regulates many myeloid cell-specific genes, including cytokine receptors for granulocyte, granulocyte-macrophage, and macrophage colony-stimulating factor, but the transcriptional cascade that underlies the cell-autonomous effects of PU.1 remains to be elucidated.

CCAAT/enhancer-binding protein ε (C/EBPε) is expressed exclusively in granuloid cells and is essential for the terminal

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differentiation of committed granulocyte progenitors (17, 34a). C/EBPε is thought to be one critical target of PML-RARA. This is strongly supported by the following observations: (i) C/EBPε is directly regulated by RARA (23); (ii) PML-RARA inhibits the expression of C/EBPε, whereas ATRA restores it (23); (iii) the introduction of C/EBPε into APL cells can mimic the ability of ATRA to drive granulocyte differentiation in vitro and repress the leukemic phenotype of APL in vivo (29); and (iv) APL cells lack secondary granules, which is the prominent characteristic of APL cells and is consistent with a deregulation of C/EBPε expression (7, 34a).

To clarify the role of PML-RARA in the pathogenesis of APL, especially how it causes the arrest of granulocytic differentiation, we focused on the biological properties of PML-RARA and how it perturbs PML function. We show for the first time that the impaired granulopoiesis in *PML*$^{-/-}$ mice is associated with the reduced expression of C/EBPε. In addition, PU.1 directly regulates C/EBPε expression, and PML modulates it by promoting the formation of a PU.1/p300 complex in an isoform-specific manner. Finally, we show that PML-RARA can block granulopoiesis by the direct transrepression of a PU.1/PML/p300 ternary complex and propose a model for how PML-RARA acts as a dominant-negative inhibitor of PML-induced transcription. These results should provide a new insight into how PML-RARA causes leukemia and should help identify new molecular targets for the treatment of APL.

MATERIALS AND METHODS

Mice and cell lines. PML-deficient mice were generated as described previously (32). Mice over 40 weeks of age were analyzed. All animals were maintained under specific-pathogen-free, temperature-controlled conditions throughout this study, in accordance with institutional guidelines. Written approval for all animal experiments was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute.

Interleukin-3-dependent myeloid L-G myeloblasts (16) and BOSC23, NIH 3T3, and HeLa cells were obtained from the Japanese Cancer Research Bank (Osaka, Japan).

Flow cytometric analysis. Bone marrow (BM) and peripheral blood cells were prepared by lysing erythrocytes in ammonium chloride buffer. In general, one million cells were incubated on ice for 45 min with the appropriate staining reagents according to standard methods. The reagents used in this study were as follows: peridinin chlorophyll protein-cyanin 5.5-conjugated streptavidin, anti-Mac-1–fluorescein isothiocyanate (M1/70-fluorescein isothiocyanate), anti-c-Kit–allophycocyanin (2B8-allophycocyanin), and anti-Gr-1–biotin (RB6-8C5-biotin). All of the reagents were purchased from Pharmingen (La Jolla, CA). Flow cytometry was performed by using a FACSCalibur apparatus (Becton Dickenson), and the results were analyzed using CELLQUEST software (Becton Dickenson).

Expression vectors. Human cDNAs encoding FLAG- or hemagglutinin (HA)-tagged PML isoforms I, II, III, IV, V, and VI were cloned into pLNCX and pLPCX retroviral mammalian expression vectors as described elsewhere previously (21). The cDNAs for PU.1, PML-RARA, PLZF-RARA, RAR, and retinoid X receptor were kindly provided by Francoise Moreau-Gachelin, Akira Kakizuka, Zu Chen, and Pierre Chambon, respectively. The deletion mutants for PU.1 and PML were constructed by PCR-mediated methods (see the supplemental material). The cDNAs for FLAG-tagged PU.1 and its deletion mutants were cloned into the metallothionein promoter-driven expression vector pMT-CB6+. The PML-RARA cDNA was cloned into the pcDNA3.1/His vector (Invitrogen, Carlsbad, CA) for Xpress tagging. HA-tagged PML-RARA cDNA was also cloned into the puromycin-resistant vector pMT-CB6+puro. HA- or FLAG-tagged p300 expression vectors were described previously (21).

Construction of L-G myeloblast clones. L-G cells (1×10^7) were transfected with 10 μg of PvuI-linearized plasmid pMT-CB6+/PU.1 (FLAG tagged) or its deletion derivatives by electroporation (960 μF, 0.35 kV). Stable clones were selected by the treatment of the cells with 1 mg/ml of G418. The expression of PU.1 was induced with 100 μM of $ZnSO_4$ and verified by Western blotting. Retroviruses were prepared from BOSC23 cells transfected with vector pLPCX encoding HA-tagged PML isoforms I to VI or C-terminal deletion mutants of PML IV, and bulk populations were selected by treatment with 0.6 μg/ml of puromycin.

Antibodies. The following antibodies were used in this study: anti-PU.1 (T-21 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (M2 mouse monoclonal; Sigma, St. Louis, MO), anti-p300 (NM11 mouse monoclonal; BD Bioscience, San Diego, CA;), anti-HA (3F10 rat monoclonal; Roche Diagnostics, Mannheim, Germany), anti-C/EBPε (C-22 rabbit polyclonal; Santa Cruz Biotechnology), anti-TFIIB (C-18 rabbit polyclonal; Santa Cruz Biotechnology), and anti-PML (PML001 rabbit polyclonal and 1B4 mouse monoclonal [MBL, Nagoya, Japan] and H-238 rabbit polyclonal [Santa Cruz Biotechnology]).

Immunoprecipitation and Western blotting. Detailed procedures for sample preparation, immunoprecipitation, and Western blotting were described elsewhere previously (21).

Immunostaining. Indirect immunofluorescence was performed as previously described (21).

EMSA. An electrophoretic mobility shift assay (EMSA) was performed according to standard procedures (see the supplemental materials for details). For supershift assays, an anti-PU.1 polyclonal antibody (Santa Cruz Biotechnology) was used.

ChIP. Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's instructions (UBI, Lake Placid, NY) by using the following primers: 5′-CCCATGAGTACCTATATGCTCA-3′ and 5′-CTCAAATCTGGC CTCCGTCACTG–3′ for region 1, 5′-AAGGCTTACATCTCTCCCTCTG-3′ and 5′-CTGTCACCCACTCCTGTGTG-3′ for region 2, and 5′-CACACGATT GTTTAGAGGTAGAAC-3′ and 5′-GAGACTTTAAGAAGCCCGTAATC-3′ for region 3.

Luciferase reporter assay. The *C/EBPε* promoter region was amplified by genomic PCR and cloned into the pGL3-Basic vector (Promega, Madison, WI) as described previously (34). The putative PU.1 binding sites were mutated by site-directed mutagenesis according to standard procedures (see the supplemental material for details). The cells were transfected with the aid of Effectene transfection reagent according to the manufacturer's instructions (QIAGEN, Valencia, CA). After 36 h, luciferase activity was determined using a Dual Luciferase assay system (Promega) according to the manufacturer's instructions. Values were normalized by the luciferase activity of a cotransfected *Renilla* luciferase-expressing vector (pRL-CMV).

RT-PCR. RNA was isolated according to standard protocols and reverse transcribed with random primer using Superscript II (Invitrogen) according to the manufacturer's instructions. For quantitative reverse transcription (RT)-PCR, the following sets of primers and internal fluorescence probes were purchased from Roche Diagnostics (Indianapolis, IN); mouse *C/EBPε* (catalog no. 04688970001), mouse *GAPDH* (catalog no. 04689089001), and mouse *PML* (catalog no. 04688996001). PCRs were performed using an ABI PRISM 7500 Fast Real-Time RCR system (Perkin-Elmer, Foster City, CA) using TaqMan Universal PCR Master Mix containing specific primers (1.2 μM) and a specific probe (0.1 μM). For conventional RT-PCR, the following sets of primers were used: 5′-GACTACAAAGACGATGACGAC-3′ (forward) and 5′-CAGTAATGGTC GCTATGGCTC-3′ (reverse) for FLAG-tagged human *PU.1* and 5′-CTTCACC ACCATGGAGAAGG-3′ (forward) and 5′-GGCATGGACTGTGGTCATGA G-3′ (reverse) for *GAPDH*.

RESULTS

Impaired granulopoiesis and reduced C/EBPε expression in PML-deficient mice. Flow cytometry analysis of sex-matched littermates revealed that circulating Gr-1^{hi} Mac-1^{+} mature granulocytes were reduced in peripheral blood from *PML*$^{-/-}$ mice (Fig. 1A), as previously described (32). On the other hand, the increase of immature granulocytes in the BM of *PML*$^{-/-}$ mice was demonstrated by a fourfold increase in Gr-1^{+} c-Kit^{+} cells (Fig. 1B). These results suggest that the terminal maturation of granulocytes is impaired in *PML*$^{-/-}$ mice. To investigate the role of PML in normal granulopoiesis, we examined the expression levels of several transcription factors that are supposed to be essential for the process. Western blotting analysis of BM mononuclear cells revealed that PML expression was reduced in proportion to the *PML* genotype.

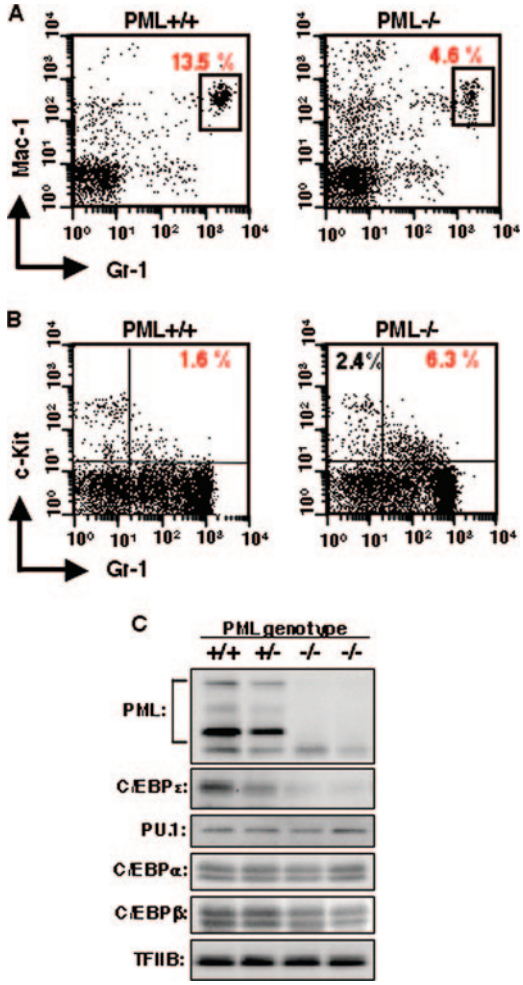


FIG. 1. Impaired granulopoiesis in *PML*$^{-/-}$ mice. (A) Mature granulocytes were reduced in peripheral blood of *PML*$^{-/-}$ mice. Whole peripheral blood cells were stained with anti-Mac-1 and anti-Gr-1 and then analyzed by flow cytometry. (B) Immature granulocytes were increased in BM of *PML*$^{-/-}$ mice. BM cells were stained with anti-Gr-1 and -c-Kit. (C) Reduced expression of C/EBPε in BM mononuclear cells from *PML*$^{-/-}$ mice. Total cell lysates were analyzed by Western blotting.

There was no significant difference in PU.1 and C/EBPα, but C/EBPβ expression was modestly reduced in *PML*$^{-/-}$ mice. On the other hand, C/EBPε expression was reduced in proportion to that of PML (Fig. 1C). These results suggest that the specific involvement of PML in C/EBPε expression may account for its underlying role in granulocytic differentiation.

***C/EBPε* is a direct transcriptional target of PU.1.** To investigate how PML is involved in C/EBPε expression, we first analyzed its promoter. The transcription of the *C/EBPε* gene is regulated mainly by the downstream Pβ promoter, which is highly conserved among different species (2, 34). The DNA sequence around the Pβ promoter is highly rich in purine tracts, which are peculiar features of myeloid cell-specific genes and often serve as potential binding sites for *ets* family transcription factors. These facts prompted us to examine if PU.1 regulates the expression of *C/EBPε*. ChIP assays of HL-60 cells revealed that PU.1 binds to the region 2, which includes the Pβ promoter, in vivo (Fig. 2A). However, we could not detect any significant binding of PU.1 to other genomic regions 4 kb upstream or downstream of the Pβ promoter (regions 1 and 3, respectively). These results indicate that PU.1 binds to a specific genomic region that includes the Pβ promoter. The specificity of this ChIP assay was further confirmed, as the same anti-PU.1 antibody failed to precipitate region 2 in MOLT-4 cells that lack endogenous PU.1 expression (see Fig. S1A in the supplemental material). EMSA detected at least two PU.1 binding regions: region A, between bp −80 and −44, and region B, between bp −40 and −18. PU.1 bound to region A more efficiently than region B (Fig. 2D, left). Inspection of the DNA sequence identified six putative *ets* core motifs (A1 to A3 and B1 to B3) (Fig. 2C). To identify the PU.1 binding sites, we constructed a *C/EBPε* promoter-containing luciferase reporter, introduced either several block mutations or various combinations of site-directed mutations into these motifs, and performed transactivation assays using NIH 3T3 cells (Fig. 2E). As we expected, the response to PU.1 was limited to a region between bp −80 and −18, which contains a purine-rich tract. Luciferase assays further revealed that the response to PU.1 was mediated between positions bp −62 and −57 (A2) and between bp −34 and −29 (B1) (Fig. 2E). Similar results were also obtained for HeLa cells (see Fig. S1B in the supplemental material), suggesting that these effects do not depend on the cell context. Competitive EMSA combined with supershift assays indicated that PU.1 specifically binds to these sites in vitro (Fig. 2D, middle and right panels). Taken together, these results show that PU.1 transactivates the *C/EBPε* gene directly.

PML IV associates with PU.1 in vivo. To investigate the interaction of endogenous PML and PU.1, an immunoprecipitation assay was performed using HL-60 cells. An anti-PML antibody raised against its N terminus successfully immunoprecipitated multiple PML isoforms (Fig. 3A) from HL-60 cell extracts and also pulled down PU.1 together with them (Fig. 3B, left). Reciprocal experiments using an anti-PU.1 antibody revealed the predominant coimmunoprecipitation of a specific PML isoform with a molecular mass of ~75 kDa (Fig. 3B, right). These results indicate the association of these endogenous proteins in myeloid lineage cells. To determine the isoform specificity for PU.1 binding, PU.1 and one of each of the PML isoforms were transiently coexpressed and subjected to immunoprecipitation. PML II and IV specifically interacted with PU.1 (Fig. 3C). An association between PU.1 and the other isoforms, including PML VI, was not successfully detected.

To further confirm those results, we next examined whether these two proteins are colocalized in cells (Fig. 3D). Immunofluorescence revealed that PU.1 was spread throughout the nucleus in NIH 3T3 cells. Upon cotransfection with PML IV, however, PU.1 formed discrete speckles and colocalized prominently with PML IV PODs. Surprisingly, and in contrast with the immunoprecipitation results, PU.1 was not recruited to PML II PODs but instead was recruited to PML VI PODs. Colocalization of PU.1 with both PML IV and VI was also observed in HeLa cells (data not shown). We further examined the subcellular localization of both proteins in mouse embryonic fibroblasts from *PML*$^{-/-}$ mice. Here, we found that PU.1 was also recruited to PML IV PODs but not to PML VI PODs

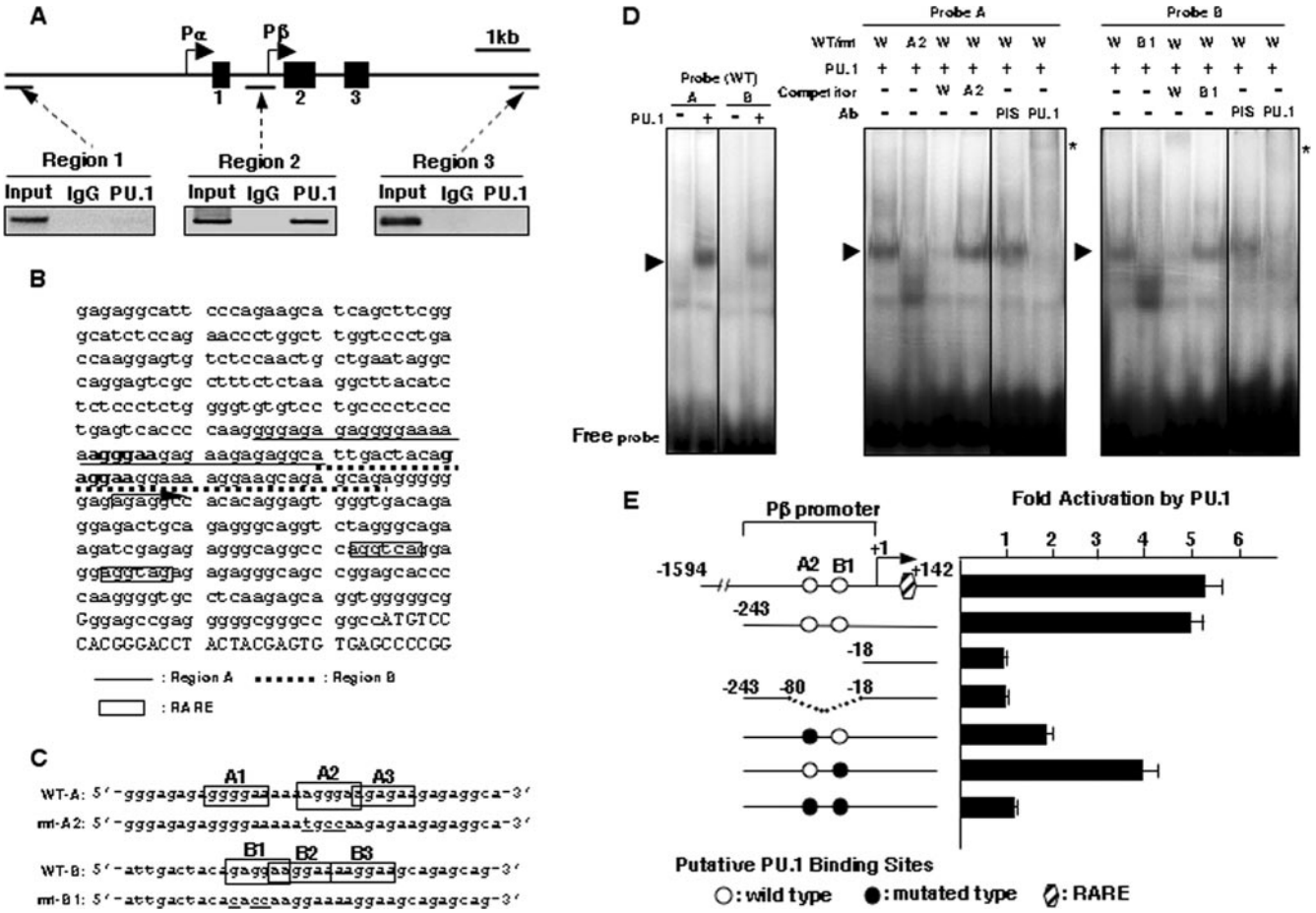


FIG. 2. PU.1 regulates the expression of the *C/EBP*ε gene. (A) A ChIP assay shows that PU.1 binds to the promoter region of the *C/EBP*ε gene in HL-60 hematopoietic cells. In the schematic of the *C/EBP*ε locus, exons are represented by boxes on the line, and transcription start sites are represented by arrows. Three regions examined for PU.1 binding are indicated. Cross-linked HL-60 chromatin was immunoprecipitated with anti-PU.1 antibody (PU.1) or isotype-matched immunoglobulin G (IgG) as a negative control. Three percent of input DNA was also PCR amplified. (B) DNA sequences of the *C/EBP*ε promoter region. A major transcriptional start site is indicated by an arrow. The oligonucleotide sequences used for EMSA probes are underlined, and the putative PU.1 binding sites are shown in boldface type. The RARE is shown in boxes. (C) Sequences of wild-type (WT) and mutated (mt) probes used for EMSA. The six putative core PU.1 binding motifs are shown in boxes, and the two PU.1 binding sites tested by EMSA are shown in boldface type. The mutated nucleotides are underlined. (D) Identification of PU.1 in DNA-protein complexes by EMSA using nuclear extracts from BOSC23 cells transfected with the PU.1 expression vector. Arrowheads indicate the PU.1-DNA complexes. Supershifted bands are indicated by asterisks. PIS, rabbit preimmune serum. (E) PU.1 response elements within the *C/EBP*ε promoter were confirmed by luciferase reporter assays. A major transcriptional start site is shown as "+1." NIH 3T3 cells were transfected with 0.1 μg of wild-type reporter plasmids containing the region between bp −1594 and +142 or bp −243 and +142 or its mutant derivatives along with 0.1 μg of a PU.1 expression vector. The results are represented as activity (*n*-fold) compared to that of PU.1 and are the average of at least three independent experiments. The error bars represent the standard deviations.

(see Fig. S2 in the supplemental material). These results suggest that the in vivo association between PU.1 and PML IV is of primary importance and that PML VI might indirectly associate with PU.1, although underlying mechanisms remain to be elucidated.

PML IV specifically cooperates with PU.1 to induce terminal differentiation of L-G myeloblasts. We next investigated the ability of each PML isoform to cooperate with PU.1 in the terminal differentiation of L-G myeloid progenitor cells (16). L-G cells were transfected with a vector for expressing PU.1 under the control of a metallothionein promoter (pMT-PU.1). We established several stable clones and confirmed that they morphologically differentiated toward polymorphonuclear cells (PMNs) upon the induction of PU.1 with $ZnSO_4$. To investigate the isoform-specific cooperation between PU.1 and PML, we used a retrovirus to transduce the L-G/MT-PU.1 cells with PML I, II, III, IV, V, VI, or mock (L-G/MT-PU.1/PML isoforms) (Fig. 4A). The level of PU.1 expression following induction with $ZnSO_4$ was the same in all seven cotransfected cells and the parent L-G/MT-PU.1 cells (see Fig. S3A in the supplemental material) (data not shown). Although the expression level of transduced PML IV seemed to be high compared to that of the endogenous one, it was almost comparable to the level of total PML expression (see Fig. S3C in the supplemental material). The induction of PU.1 expression alone retarded cell growth, and the coexpression of PML IV enhanced this effect (Fig. 4B). PU.1 and PML IV also had a synergistic effect on morphological differentiation (Fig. 4C).

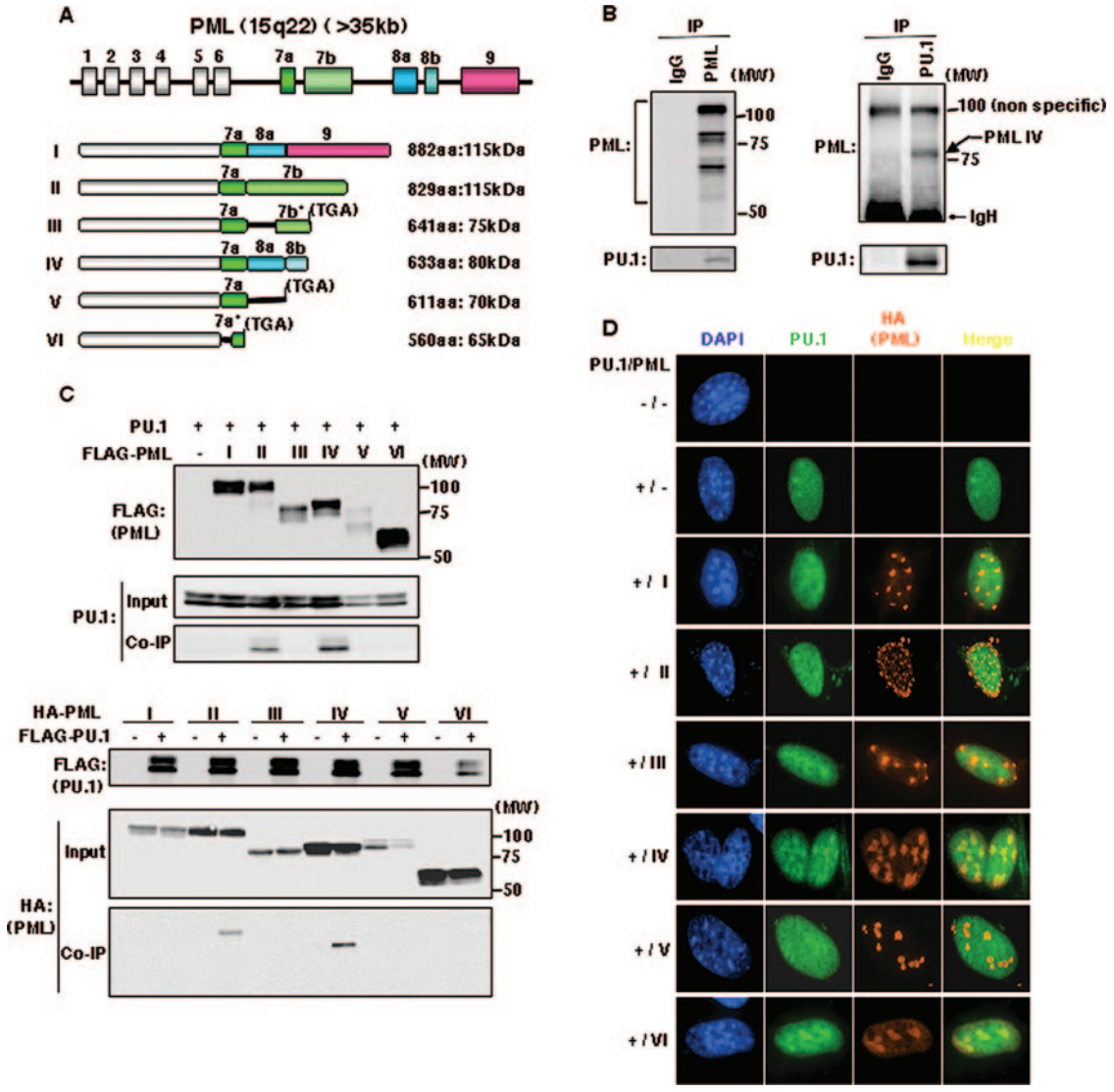


FIG. 3. PML associates with PU.1 in vivo. (A) Schematic illustration of the genomic structure of the *PML* gene. Boxes represent exons, and their exon numbers are indicated. Six major alternatively spliced isoforms are shown. The solid lines indicate retained introns. Asterisks show frameshifts of the coding sequence compared to PML II. Numbers of amino acid (aa) residues and the apparent molecular masses of each isoform are given. (B) Association of endogenous PU.1 and PML. Total cell lysates from HL-60 cells were immunoprecipitated with an anti-PML (left) or an anti-PU.1 (right) antibody and then analyzed by Western blotting. Note that PML, with a molecular mass of ~75 kDa, was coprecipitated predominantly with PU.1. IgG, immunoglobulin G. (C) PU.1 coimmunoprecipitates with PML II and IV. Total cell lysates from BOSC23 cells transfected with the indicated expression vectors were subjected to immunoprecipitation with an anti-FLAG antibody and then analyzed by Western blotting. The antibodies used for Western blotting are indicated on the left of each panel. IP, immunoprecipitates; MW, molecular weight (in thousands). (D) PU.1 and PML IV were colocalized within PODs. The expression vectors indicated were transiently coexpressed in NIH 3T3 cells and then costained with antibodies to PU.1 and HA (for PML). DAPI, 4′,6′-diamidino-2-phenylindole.

After 7 days of culture with $ZnSO_4$, most of the control mock-transfected cells (L-G/MT-PU.1/mock) differentiated around the metamyelocyte stage, and only a few mature PMNs were observed. In contrast, more than 60% of L-G/MT-PU.1/PML IV cells differentiated into mature PMNs. The PML VI isoform cooperated moderately with PU.1 to induce granulocytic differentiation (see Fig. S3D in the supplemental material). The other PML isoforms (I, II, III, and V), however, did not affect PU.1-induced differentiation of L-G cells (data not shown). It is noteworthy that the cooperativity of PU.1 and PML isoforms in granulocytic differentiation was comparable to their POD colocalization capability.

Next, we examined the effect of PML IV on PU.1 transcription activity. Luciferase reporter assays showed that among six PML isoforms, only PML IV had a marked effect on the activation of the *C/EBPε* reporter by PU.1 (Fig. 4D). A parallel experiment using a reporter of the *M-CSFR* promoter also demonstrated a specific cooperation between PU.1 and PML IV, indicating that the interaction between these two proteins does not depend on the promoter context (see Fig. S3E in the supplemental material).

Moreover, we next examined whether PML IV could affect the expression of endogenous C/EBPε during PU.1-induced granulocytic differentiation (Fig. 4E). In L-G/MT-PU.1/mock

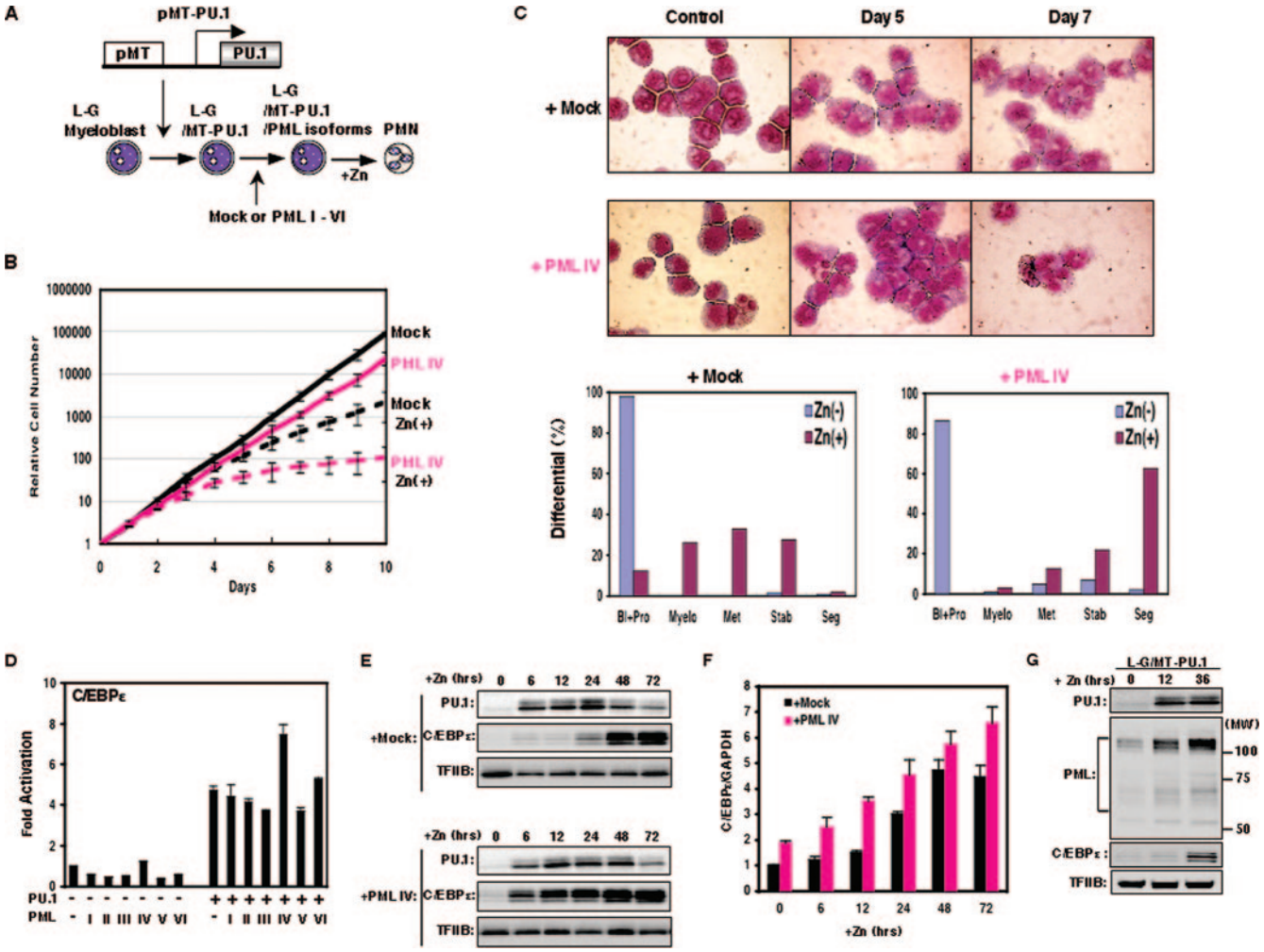


FIG. 4. PML IV and PU.1 cooperate to accelerate terminal differentiation of L-G myeloblasts. (A) Construction of L-G cells transfected with a plasmid encoding PU.1 under the control of the metallothionein promoter (pMT-PU.1). Stable clones could be induced to differentiate into PMNs. The cells were further transformed with each PML isoform or mock using a retroviral vector, generating L-G/MT-PU.1/PML I to VI or mock, respectively. (B) Upon expression of PU.1, PML IV synergistically suppressed the proliferation of L-G cells. (C) Differentiation of L-G cells upon induction of PU.1 by $ZnSO_4$ treatment. Cytospin-prepared cells were stained with May-Giemsa stain (top) and evaluated by morphological criteria after 7 days (bottom). Bl, blast; Pro, promyelocyte; My, myelocyte; Met, metamyelocyte; Stab, stab cell; Seg, PMNs. (D) PML IV specifically enhances PU.1-induced activation of the *C/EBPε* promoter-containing luciferase reporter in NIH 3T3 cells. The effector plasmids are indicated. (E) Western blotting shows that PML IV and PU.1 synergistically enhance the expression of C/EBPε in L-G cells. (F) Real-time RT-PCR was used to quantify *C/EBPε* mRNA in L-G/MT-PU.1/mock and PML IV cells treated with $ZnSO_4$ for the indicated times. All results are given in relative units compared to *GAPDH*. Result are means ± standard deviations of triplicate determinations of a representative experiment. Note that PCR detects all *C/EBPε* mRNA isoforms generated by the alternative use of promoters or splicing. (G) Western blotting shows that the expression of endogenous PML protein increases during PU.1-induced granulocytic differentiation. MW, molecular weight (in thousands).

cells, C/EBPε expression started to increase 24 h after exposure to $ZnSO_4$, and it reached a maximum after 48 to 72 h. On the other hand, the coexpression of PML IV enhanced C/EBPε expression within 6 h after $ZnSO_4$ treatment in parallel with PU.1 expression. The PML VI isoform modestly promoted PU.1-induced C/EBPε expression. The other PML isoforms (I, II, III, and V), however, did not affect PU.1-induced expression of C/EBPε (see Fig. S3F in the supplemental material).

To confirm that PML IV enhancement of C/EBPε expression was due to transcriptional activation, quantitative RT-PCR was performed (Fig. 4F). In L-G/MT-PU.1/PML IV cells, all six time points showed elevated *C/EBPε* transcripts compared to L-G/MT-PU.1/mock cells. The difference was more prominent before C/EBPε expression started to increase in L-G/MT-PU.1/mock cells.

We next investigated why more than 12 h was required before the induction of C/EBPε expression in LG/MT-PU.1 cells in spite of possible direct regulation by PU.1. Western blots showed that PU.1 induced the expression of endogenous PML in L-G/MT-PU.1 cells (Fig. 4G). PML expression did not increase after $ZnSO_4$ treatment in either parent L-G cells or L-G/MT-PU.1 cells driven to differentiate into granulocytes by treatment with granulocyte colony-stimulating factor instead of interleukin-3 (see Fig. S3G in the supplemental material). These results indicate that endogenous PML expression is specifically regulated by PU.1. Since quantitative

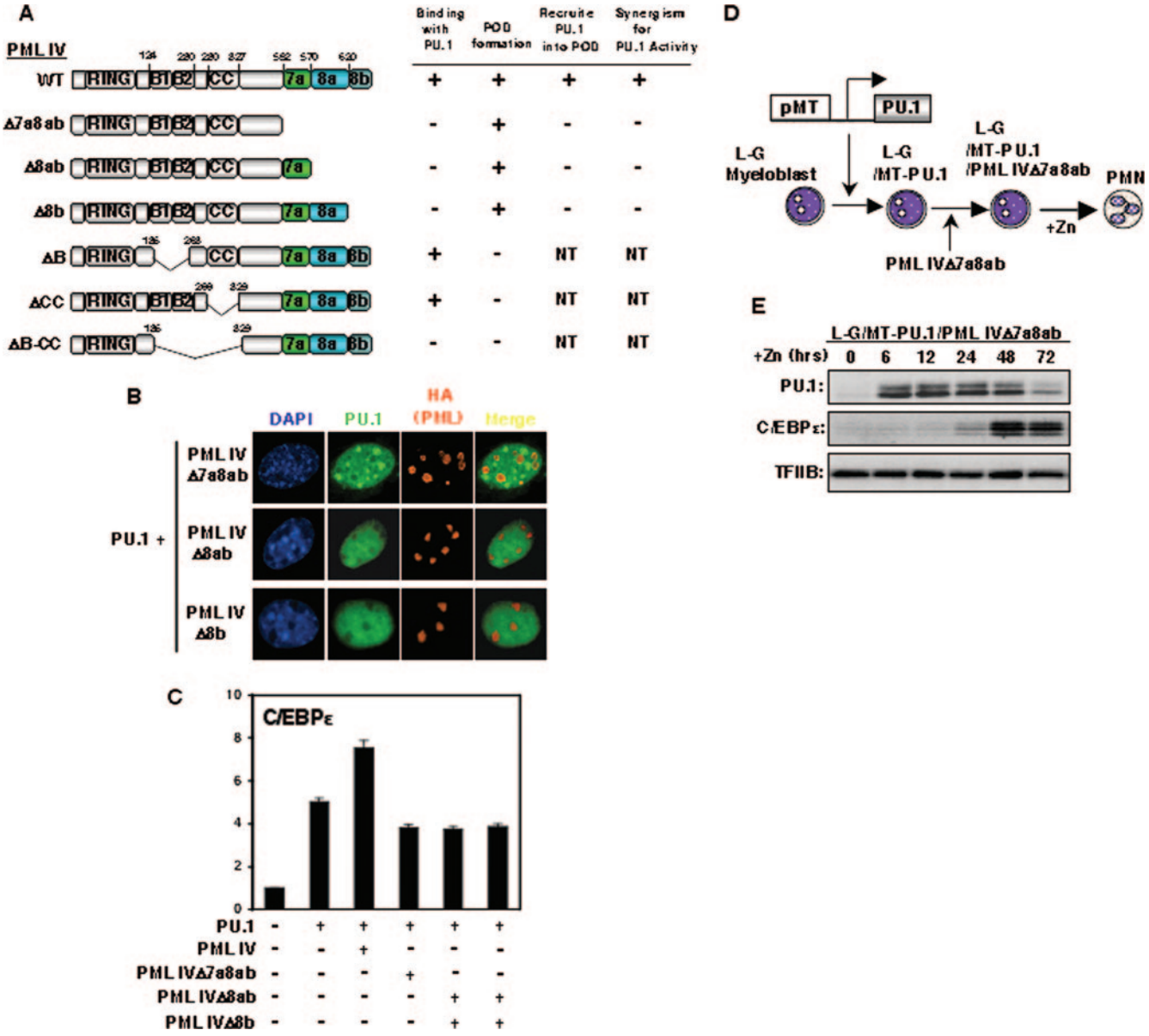


FIG. 5. Enhancement of PU.1-induced terminal differentiation of L-G cells requires the C terminus of PML IV. (A) Schematics of the PML IV mutants and summary of the domain mapping of the physical and functional interaction with PU.1. Pro, proline-rich region; RING, RING finger domain; B1 and B2, B boxes; CC, coiled-coil domain; WT, wild type; NT, not tested. (B) Immunofluorescence shows that the PML IV C-terminal deletion mutants do not colocalize with PU.1 in NIH 3T3 cells. DAPI, 4′,6′-diamidino-2-phenylindole. (C) Luciferase reporter assays show that PML IV C-terminal deletion mutants do not enhance PU.1-induced transcription in NIH 3T3 cells. (D) Schematics of the construction of PU.1-inducible L-G cells transduced with a PML IV C-terminal deletion mutant (L-G/MT-PU.1/PML IVΔ7a8ab) or mock (L-G/MT-PU.1/mock). (E) Western blots show that the PML IVΔ7a8ab is unable to enhance PU.1-induced expression of C/EBPε in L-G cells.

RT-PCR analysis revealed that endogenous *PML* mRNA also increased (data not shown), PU.1 regulates PML expression, at least in part, at the transcriptional level. An important finding is that C/EBPε expression was also induced in a fashion parallel to that of PML expression in L-G/MT-PU.1 cells (Fig. 4G). Together with the finding that the induction of C/EBPε becomes much faster when PML IV is already expressed exogenously (see Fig. S3H in the supplemental material), these results clearly indicate that PU.1 action on *C/EBPε* transcription is modulated by PML.

Structure-function relationship of the PU.1-PML IV interaction and its relevance in myeloid terminal differentiation. We performed coimmunoprecipitation assays to determine the region of PML required for the association with PU.1. Deletion of the C-terminal 13 amino acids of PML IV, which corresponds to the isoform-specific exon 8b, completely abolished the formation of the PU.1-PML complex (Fig. 5A) (see Fig. S4A in the supplemental material). The integrity of B boxes and the coiled-coil region was also required for an association with PU.1 (Fig. 5A) (see Fig. S4A in the supplemental mate-

rial). In addition, these C-terminally-deleted PML mutants could no longer recruit PU.1 to PODs in vivo (Fig. 5B), nor could they enhance PU.1-mediated transcription (Fig. 5C). Because the deletion mutant lacking exon 8b was unstable, and another one lacking exons 8a and 8b (PML IVΔ8ab) was not efficiently expressed in L-G cells (see Fig. S4B and S4C in the supplemental material), we used L-G/MT-PU.1 cells expressing PML lacking exons 7a, 8a, and 8b (PML IVΔ7a8ab) for further analysis (Fig. 5D). In addition to losing the colocalization and transcriptional cooperation with PU.1, PMLΔ7a8ab had no effect on the profile of PU.1-induced C/EBPε expression and cell differentiation (compare Fig. 5E and S4D and S4E in the supplemental material with Fig. 4B, C, and E).

We also performed reciprocal experiments employing PU.1 mutants. Coimmunoprecipitation analysis using PU.1 deletion mutants showed that the acidic amino-acid-rich region (DE region) of the PU.1 transactivation domain is necessary for an association with PML IV (Fig. 6A) (see Fig. S5A in the supplemental material). As expected, PML IV could no longer recruit PU.1 lacking the DE region (PU.1ΔDE) to PODs in vivo (Fig. 6B, top), nor could it enhance PU.1ΔDE-mediated transcription, although PU.1ΔDE itself has a weak ability to enhance transcription (Fig. 6C, lanes 5 and 6). In agreement with these results, the expression of PU.1ΔDE did not effectively induce the expression of C/EBPε, and the overexpression of PML IV could not rescue the expression of C/EBPε (Fig. 6E, top) or significantly affect the differentiation of L-G cells (see Fig. S5D and S5E in the supplemental material).

To confirm the significance of the physical interaction between PU.1 and PML IV, we performed parallel experiments using a PU.1ΔPEST mutant that retains the ability to bind PML IV (see Fig. S5A in the supplemental material). In contrast to PU.1ΔDE, PML IV enhanced PU.1ΔPEST-mediated transcription to an extent similar to that of wild-type PU.1 (Fig. 6C, lanes 7 and 8). PML IV also enhanced C/EBPε expression in L-G/MT-PU.1ΔPEST cells, although it did not affect the time course of C/EBPε expression (Fig. 6E, bottom). This cooperation between PML IV and PU.1ΔPEST was also observed in the granulocytic differentiation of L-G cells (see Fig. S5H and S5I in the supplemental material). Interestingly we noticed that PU.1ΔPEST expression in PML IV-transduced cells was maintained at a high level even after 48 h of treatment of $ZnSO_4$ compared to mock-transduced cells. RT-PCR analysis revealed that mRNA expression of PU.1ΔPEST was equal in both cells (see Fig. S5J in the supplemental material). These results suggest that PML IV enhances PU.1ΔPEST expression by a posttranscriptional mechanism.

Taken together, these results demonstrate that the specific interaction between PU.1 and PML IV is involved in their abilities to promote granulocytic differentiation.

PML IV promotes the association of PU.1 and p300 during granulocytic differentiation to form complexes for active expression of C/EBPε. We next investigated the significance of the interaction of PU.1 and PML in regulating C/EBPε expression during granulocytic differentiation. In HL-60 cells, RA treatment immediately increased the expression of PU.1, PML, and p300 for 48 h (Fig. 7A), which thereafter decreased (data not shown). The expression of all PML isoforms increased evenly. C/EBPε expression markedly increased for 48 h, whereas C/EBPβ expression transiently increased and then returned to a level equal to that of untreated cells. To determine the interaction of the ternary complex of PU.1/PML/p300 on the *C/EBP*ε promoter, ChIP analysis was performed (Fig. 7B). Upon RA treatment, promoter-associated PU.1 modestly increased, and PML association gradually increased. A rapid recruitment of p300 within 24 h of RA treatment, which may be mediated by promoter-bound RAR through an RA-responsive element (RARE), was followed by further accumulation after 48 h of treatment. Note that the amount of p300 that coimmunoprecipitated with PU.1 was only minimally detected in untreated HL-60 cells but significantly increased within 48 h by RA treatment, and this increase was proportional to the amount of PML coimmunoprecipitation rather than the amount of PU.1 itself (Fig. 7C). These results demonstrate that the ternary complex of PU.1/PML/p300 forms on the *C/EBP*ε promoter and that the association of the complex increases in parallel to PML recruitment on the promoter during the early stage of granulocytic differentiation.

PU.1 alone induced C/EBPε expression, although relatively slowly, in L-G cells (Fig. 4E and 7D). An interesting finding is that C/EBPε expression was induced proportionally to p300 coimmunoprecipitation with PU.1. Another important finding is that the amount of p300 coimunnoprecipitation with PU.1 increased proportionally to the expression level of PML (Fig. 7D). These results suggest a scaffold function of PML in the association of PU.1 and p300. To confirm this, the association of these proteins was further examined by immunoprecipitation experiments using a transient expression system. Although the interaction between p300 and PU.1 is seemingly enhanced by the coexpression of PML IV (Fig. 7E, top panels, lanes 3 and 4), the coexpression of PML IV also increased PU.1 expression. These results raised the possibility that the increased coimmunoprecipitation of p300 may be due simply to an increased expression and availability of PU.1. To exclude this possibility, a reciprocal experiment was performed. Coimmunoprecipitation of PU.1 with p300 was not efficiently detected in the absence of PML IV but was easily observed when PML IV was coexpressed (Fig. 7E, middle panels, lanes 3 and 4). On the other hand, the coexpression of PU.1 did not affect the interaction between p300 and PML IV (Fig. 7E, bottom panels, lanes 3 and 4). These results suggest that the association of PU.1 and p300 is more labile than that of PML and p300 and support the data obtained in HL-60 cells showing that it can be stabilized by PML IV. We next performed immunofluorescence experiments to further confirm whether PU.1, p300, and PML form ternary complexes in vivo (Fig. 7F). Whereas both PU.1 and p300 localized throughout the nucleus, they were concentrated in PODs when PML IV was coexpressed. We then investigated the cooperation of PML IV and p300 in PU.1-induced transcription by luciferase reporter assays (Fig. 7G). PU.1 activation of the *C/EBP*ε promoter was only slightly enhanced by the coexpression of p300 alone, but it was synergistically enhanced by the coexpression of PML IV and p300.

Notably, PU.1 was not efficiently recruited to abnormal nuclear aggregates of a sumoylation-deficient PML IV-3R mutant (see Fig. S6A in the supplemental material), even though PML IV-3R could still associate with PU.1 in immunoprecipitation experiments (data not shown). In contrast, p300 still efficiently colocalized with PML IV-3R. In agreement with its inability to recruit PU.1, PML IV-3R did

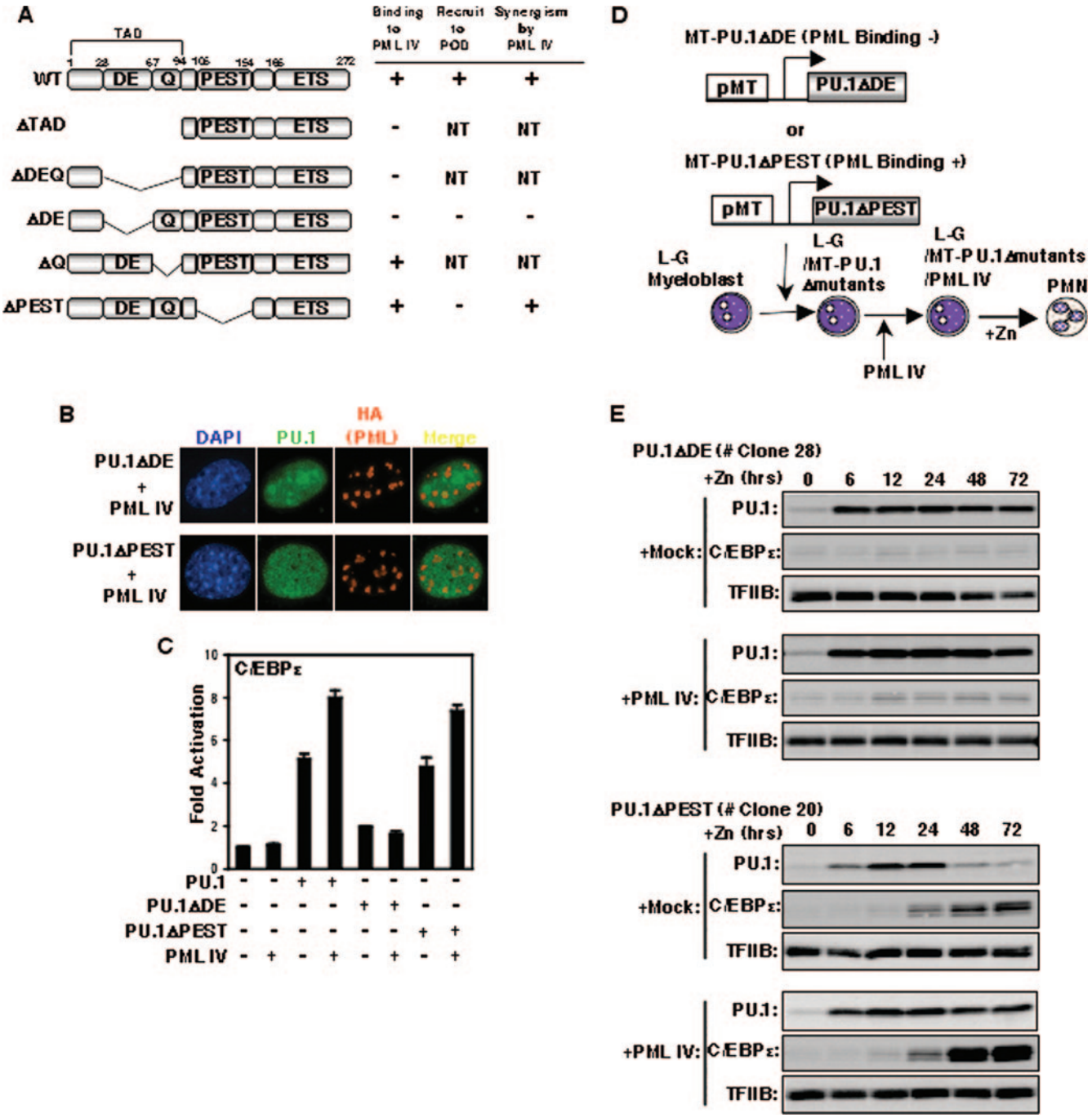
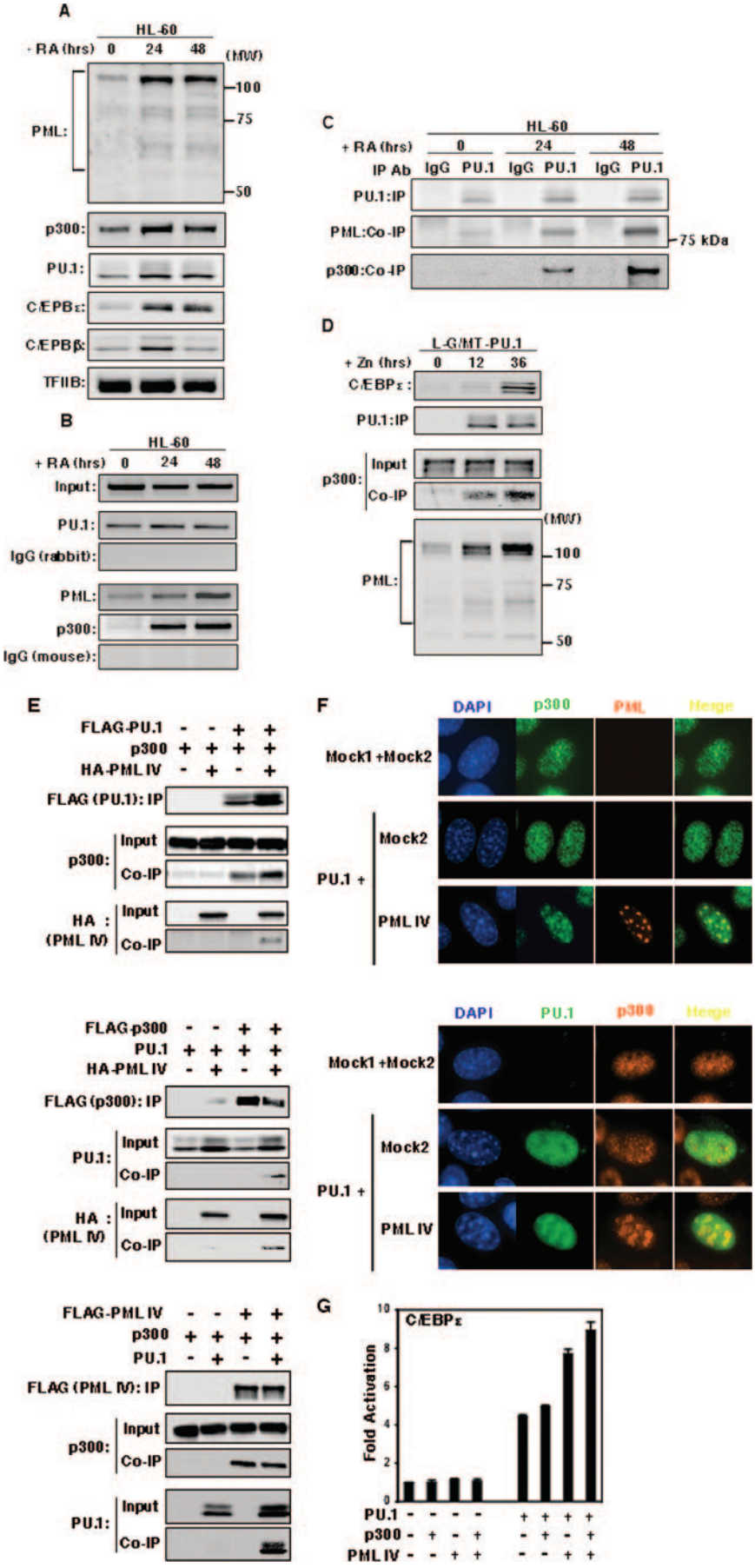


FIG. 6. The PU.1 transactivation subdomain is required for the enhancement of terminal differentiation by PML IV in L-G cells. (A) Schematics of PU.1 mutants and summary of the domain mapping of the physical and functional interaction with PML IV. TAD, transactivation domain; DE, acidic amino acid-rich region; Q, glutamine-rich region; ETS, *ets* DNA-binding domain; WT, wild type; NT, not tested. (B) Immunofluorescence shows that the PU.1 mutants do not colocalize with PML IV in NIH 3T3 cells. DAPI, 4′,6′-diamidino-2-phenylindole. (C) Luciferase reporter assays show that PML IV does not enhance the induction of transcription by the PU.1ΔDE. (D) Schematics of the L-G myeloblast clones transduced with mutant forms of PU.1. Those cells were further transduced with PML IV (L-G/MT-PU.1 Δmutants/PML IV) using a retroviral vector. (E) Western blots show that PML IV enhances the induction of C/EBPε expression by PU.1ΔPEST but not by PU.1ΔDE.

not enhance PU.1 transactivation (see Fig. S6B in the supplemental material). These results suggest that a normal POD structure would be crucial for transcriptional synergism between PU.1 and PML IV.

PML-RARA disrupts active PU.1/PML/p300 transcriptional ternary complex. We next tested whether PML-RARA can affect PU.1-induced transcription. The *C/EBPε* promoter contains RARE. We found that whereas ligand-unbound RARA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HL-60
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PML:
100
75
50
p300:
PU.1:
C/EPBε:
C/EPBβ:
TFIIB:
B
HL-60
+ RA (hrs) 0 24 48
Input:
PU.1:
IgG (rabbit):
PML:
p300:
IgG (mouse):
C
HL-60
+ RA (hrs) 0 24 48
IP Ab IgG PU.1 IgG PU.1 IgG PU.1
PU.1:IP
PML:Co-IP
75 kDa
p300:Co-IP
D
L-G/MT-PU.1
+ Zn (hrs) 0 12 36
C/EBPε:
PU.1:IP
p300:
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Co-IP
(MW)
PML:
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50
E
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p300 + + + +
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FLAG (PU.1): IP
p300:
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Co-IP
HA : (PML IV)
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FLAG-p300 - - + +
PU.1 + + + +
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Mock1 +Mock2
PU.1 +
Mock2
PML IV
DAPI PU.1 p300 Hoege
Mock1 +Mock2
PU.1 +
Mock2
PML IV
G
C/EBPε
Fold Activation
0
2
4
6
8
10
PU.1 - - - - + + + +
p300 - + - + - + - +
PML IV - - + + - - + +

represses *C/EBP*ε promoter activation, RA releases it to allow PU.1 to transactivate *C/EBP*ε expression (see Fig. S7A in the supplemental material). PML-RARA also repressed promoter activity in the absence of RA (Fig. 8A). We also found that the enhancement of PU.1-induced transcription by PML IV was greatly reduced by the coexpression of a much lower amount of PML-RARA, suggesting its potent dominant-negative effect on PML IV. In addition, PML-RARA repressed the transactivation by PU.1 even in the absence of exogenous coexpression of PML IV.

To determine whether the direct recruitment of PML-RARA to the promoter is required for its inhibitory effect, we performed transactivation experiments using the RARE-mutated (*C/EBP*ε*-mtRARE*) reporter to which PML-RARA could no longer bind. Neither PML-RARA nor the RARA/retinoid X receptor affected the reporter activity in the absence of RA (data not shown); however, PML-RARA still dose-dependently inhibited both PU.1 transactivation and the PML IV enhancement of PU.1-induced transcription, similar to the wild-type reporter (Fig. 8B). These results suggest that the inhibition of *C/EBP*ε expression by PML-RARA is caused by the targeting of the PU.1-transcription factor complex. The *M-CSFR* promoter is also transrepressed by PML-RARA (data not shown), indicating that the effect of PML-RARA does not depend on the promoter context. Furthermore, PLZF-RARA, another APL-related chimera, disrupts the normal POD structure (20) and has effects that are similar to those of PML-RARA (see Fig. S7B in the supplemental material). These results suggest that the POD structure is required for PU.1 transactivation and is targeted by both chimeras.

To examine the inhibitory effects of PML-RARA on a different class of transcription factors, we performed parallel experiments using AML1b, which is functionally modulated by PML I (Fig. 8C). In contrast to its effects on PU.1, PML-RARA only partially attenuated the PML I enhancement of AML1b transcription. To exclude the possibility that the inhibitory action of PML-RARA is directed towards PML IV function, another parallel experiment was performed. c-Myb was also associated with and superactivated by PML IV but not markedly affected by PML-RARA (data not shown). These results indicate that PML-RARA specifically targets the interaction of PU.1 and PML IV.

We next considered the underlying mechanism for the differences in the effects of PML-RARA on PU.1 and AML1b. Immunoprecipitation experiments revealed that in PU.1 immunoprecipitates, the amount of p300 was remarkably reduced and that PML IV was lost when PML-RARA was coexpressed (Fig. 8D, top). In contrast, the coexpression of PML-RARA did not affect the amount of p300 coprecipitation but completely dissociated PU.1 from the PML IV immunoprecipitates (see Fig. S7C in the supplemental material). On the other hand, analysis of the AML1b complex revealed that the amount of p300 coprecipitation was not affected by the coexpression of PML I and/or PML-RARA (Fig. 8D, lower panels). The most striking difference was that PML-RARA coprecipitated with AML1b in the presence of PML I. Immunofluorescence analyses agreed with these results. PU.1 and AML1b were specifically colocalized in PML IV PODs and PML I PODs, respectively. When PML-RARA was coexpressed, the POD structures were disrupted, and PU.1 no longer colocalized with the PML IV microspeckles (Fig. 8E, top), whereas AML1b still colocalized with PML I microspeckles (Fig. 8E, bottom).

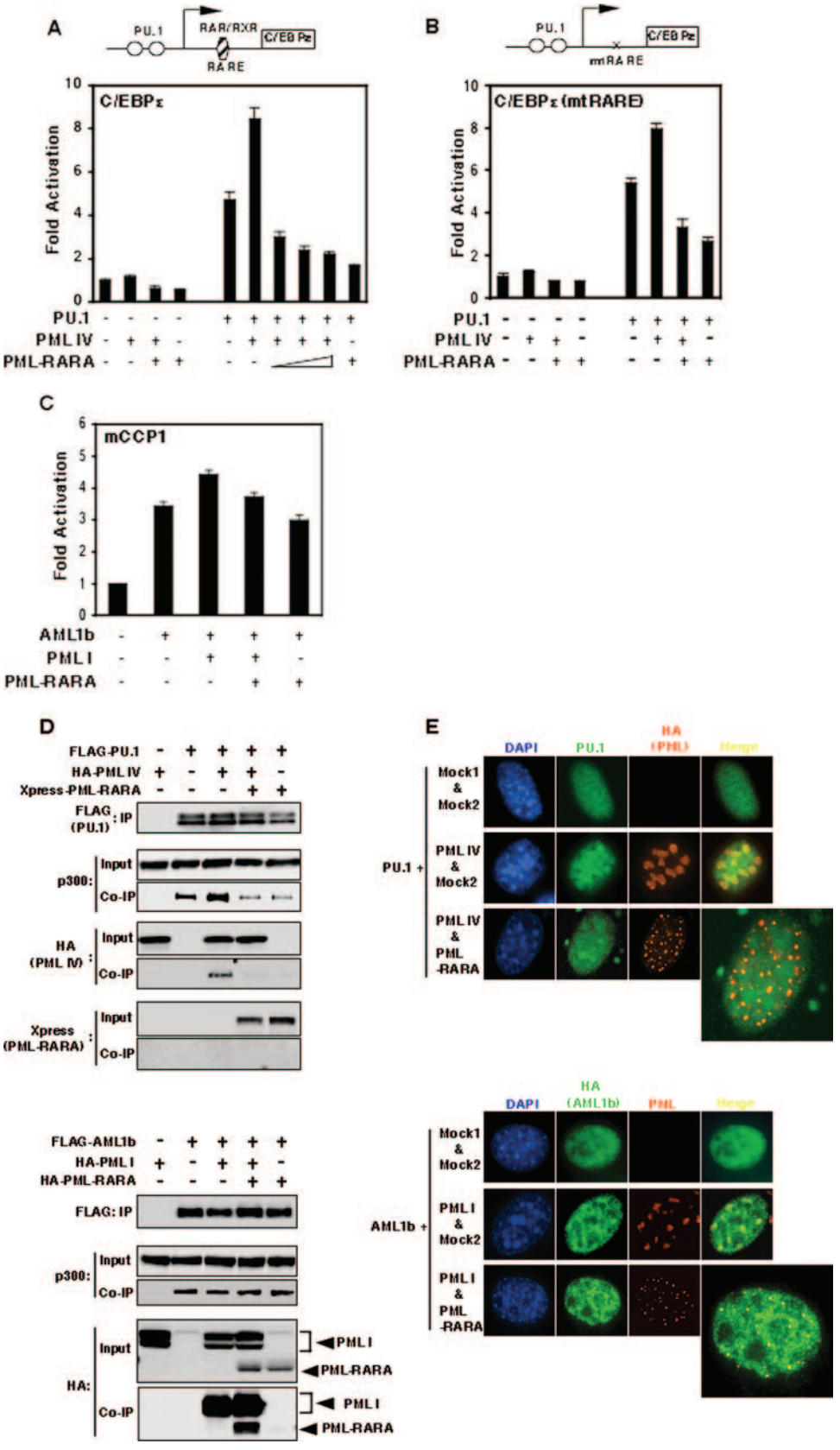
Finally, we examined the inhibition of PU.1-mediated granulocytic differentiation by PML-RARA in L-G/MT-PU.1 cells. The expression of PML-RARA in these cells (L-G/MT-PU.1/MT-PML-RARA) markedly suppressed PU.1-induced C/EBPε expression (Fig. 8F). The induction of PU.1 expression never reduced cell proliferation in those cells (data not shown). Morphological examination revealed that the expression of PML-RARA caused L-G cells to take on the appearance of APL cells and eliminated the ability of PU.1 to cause granulocytic differentiation, resulting in a premature arrest of differentiation (Fig. 8G).

DISCUSSION

In this study, we investigated the role of PML in myeloid differentiation and how the dominant-negative PML-RARA fusion affects the normal function of PML and gives rise to APL. Our results indicate the following: (i) PML cooperates with PU.1 to regulate C/EBPε expression during normal myeloid development, (ii) PML promotes the formation of an active transcription factor complex of PU.1 and p300 on the *C/EBP*ε promoter during granulocytic differentiation, and (iii) PML-RARA has a dominant-negative effect not only on RA signaling but also on PML-induced transcription by disrupting the PU.1/PML/p300 ternary complex.

Role of PML in granuloid differentiation. PML is essential for RA action to induce terminal myeloid differentiation of precursor cells (32). On the other hand, the role of RA signaling during myeloid development is still controversial. Although C/EBPε is one of the most promising targets to help elucidate

FIG. 7. PML enhances the formation of the PU.1/p300 complex. (A) Expression of the PU.1/PML/p300 complex and selected C/EBP family members in HL-60 cells treated with RA for the indicated times. MW, molecular weight (in thousands). (B) PML and p300 are increasingly recruited onto the *C/EBP*ε promoter in HL-60 cells treated with RA during the early stage of granulocytic differentiation. ChIP assays for region 2 were performed using antibodies as indicated. (C) The PU.1/p300 complex increases during RA-induced granulocytic differentiation. Lysates from HL-60 cells treated with RA for the indicated times were immunoprecipitated (IP) with an anti-PU.1 antibody (Ab), and coprecipitation of PML and p300 was analyzed by Western blotting. IgG, immunoglobulin G. (D) The PU.1/p300 complex increases along with PU.1 granulocytic differentiation. Lysates from L-G/MT-PU.1 cells treated with $ZnSO_4$ for the indicated time were immunoprecipitated with an anti-FLAG antibody (for PU.1 precipitation) and analyzed by Western blotting. The expression of C/EBPε and the PML protein is also shown. (E) Lysates from BOSC23 cells transfected with the indicated expression vectors were analyzed by immunoprecipitation with an anti-FLAG antibody and by Western blotting. (F) PML IV causes PU.1 and p300 to colocalize within PODs. Immunofluorescence was performed using anti-p300 and anti-PML (top) or an anti-PU.1 and anti-p300 (bottom) antibodies in NIH 3T3 cells transiently expressing PU.1 and PML IV. DAPI, 4′,6′-diamidino-2-phenylindole. (G) Functional relevance of PML IV effects on the PU.1/p300 complex. Luciferase assays using a reporter containing the *C/EBP*ε promoter were performed using NIH 3T3 cells.


A
PU.1
RAR/RXR
C/EB Pε
RARE
C/EBPε
Fold Activation
PU.1
PML IV
PML-RARA
B
PU.1
C/EB Pε
mtRARE
C/EBPε (mtRARE)
Fold Activation
PU.1
PML IV
PML-RARA
C
mCCP1
Fold Activation
AML1b
PML I
PML-RARA
D
FLAG-PU.1
HA-PML IV
Xpress-PML-RARA
FLAG (PU.1): IP
p300: Input
Co-IP
HA (PML IV): Input
Co-IP
Xpress (PML-RARA): Input
Co-IP
FLAG-AML1b
HA-PML I
HA-PML-RARA
FLAG: IP
p300: Input
Co-IP
HA: Input
PML I
PML-RARA
Co-IP
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DAPI
PU.1
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PU.1 +
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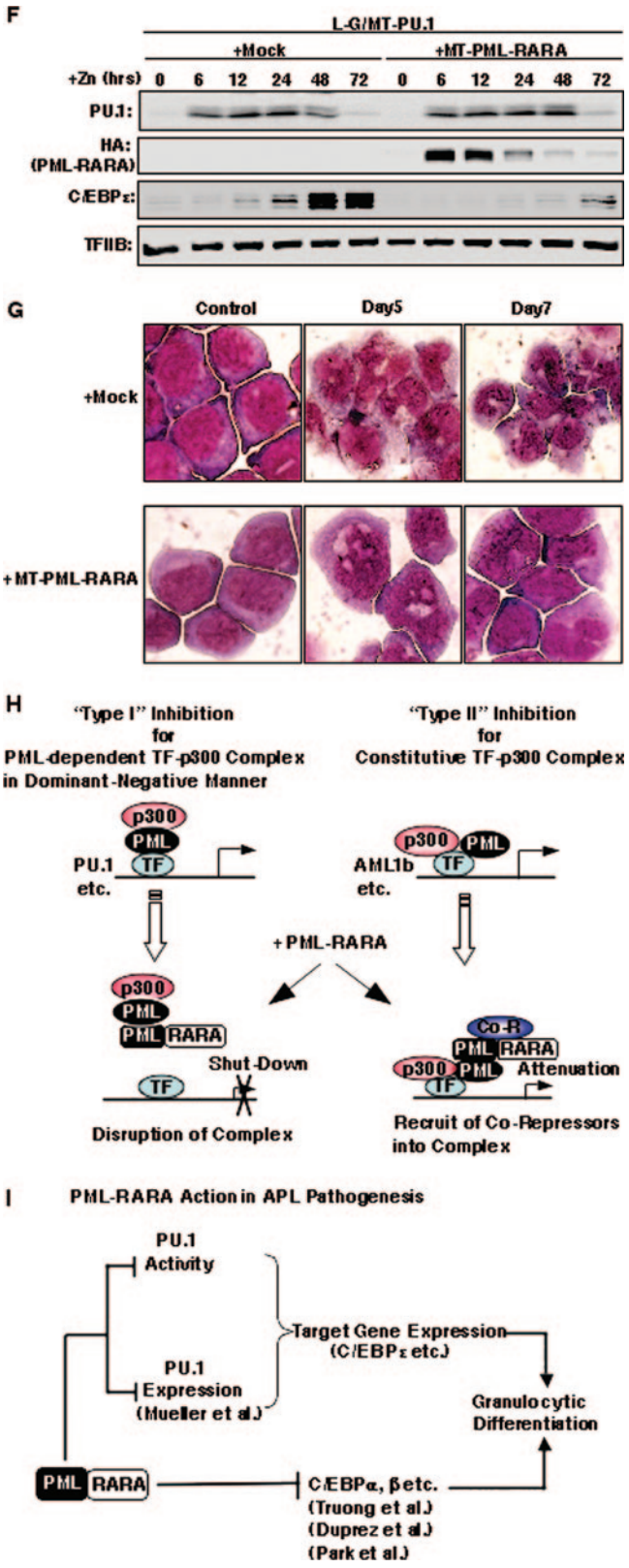

FIG. 8. PML-RARA disrupts PU.1/p300 complexes and prevents the enhancement of PU.1-induced C/EBPε expression by PML. (A) Luciferase assays in NIH 3T3 cells show that PML-RARA inhibits both the PU.1-mediated transactivation of *C/EBPε* and the enhancement of its transcription by PML IV in a dominant-negative fashion. (B) RARE is dispensable for PML-RARA-mediated inhibition of both PU.1- and PML IV-enhanced expression of *C/EBPε*. (C) PML-RARA has little effect on AML1b-mediated transcription. (D) PML-RARA disrupts the PML/PU.1/p300 complex but not the PML/AML1b/p300 complex. Lysates from BOSC23 cells transfected with the indicated expression vectors were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blotting. Note that a 20-fold-longer exposure was needed to detect the coprecipitation of p300 with PU.1 than was needed to detect the coprecipitation with AML1b. (E) Differential effects of PML-RARA on the POD colocalization of transcription factors. PML-RARA disrupts PODs, resulting in APL-associated microspeckle structures. PU.1 was lost from these structures, whereas AML1b remained. DAPI, 4′,6′-diamidino-2-phenylindole. (F) Western blots show that PML-RARA potently suppressed the ability of PU.1 to induce C/EBPε expression in L-G cells. (G) PML-RARA potently suppresses PU.1-induced granulocytic differentiation of L-G cells according to morphological criteria. (H) Model of the inhibitory mechanisms of PML-RARA towards different classes of transcription factor complexes. In type I inhibition (e.g., for the PU.1 complex), PML-RARA has a dominant-negative effect. In contrast, in type II inhibition (e.g., for the AML1b complex), PML-RARA only attenuates the activity. (I) Model of PML-RARA-mediated differentiation arrest.

the RA action in granulopoiesis (23), *RARA*$^{-/-}$ mice normally express C/EBPε and show normal granulopoiesis. Rather, neutrophil differentiation occurs faster for BM cells derived from *RARA*$^{-/-}$ mice than for those derived from wild-type mice (11). These observations suggest that RARA is dispensable for granulopoiesis, and so the role of PML is other than to modulate the RA signaling. Granulopoiesis seems to be controlled by two pathways, at least in vitro, finally accompanying the increased expression of C/EBP families (37). Insufficient granulopoiesis observed in *PML*$^{-/-}$ mice would be explained by the redundancy of the process itself or that of C/EBPβ and C/EBPε function in addition to the regulatory rather than the mandatory function of PML. Contrary to data from a previous study (32), we found that immature granulocytes increase in BM of *PML*$^{-/-}$ mice. This discrepancy may be due to a different set of antibodies used for flow cytometry, which successfully revealed this subtle difference, which was undetected by the differential counts on cytospin smears.

Since PU.1 expression can induce L-G cells to differentiate into mature granulocytes without any additional cytokines, it is likely that PU.1 activates an unrevealed transcription cascade(s) that directs terminal differentiation in a cell-autonomous manner. Although the Pβ promoter contains RARE, and *C/EBPε* is upregulated by a pharmacological dose of RA, at least in vitro (23), RA treatment does not affect the DNase I hypersensitivity of Pβ (14). Those findings imply that a *trans*-acting factor(s) other than the RAR would control the chromatin structure. Another interesting finding is that RA fails to induce the expression of C/EBPε in C/EBPα-deficient cells (37), suggesting that a C/EBPα-initiating transcription cascade is responsible for RA signaling. Since PU.1 is one of the target genes induced by C/EBPα (31), it would be reasonable to speculate on the possible involvement of PU.1 in the regulation of *C/EBPε*. Taking our results of *C/EBPε* Pβ promoter analysis together with in vivo observations of *RARA*$^{-/-}$ mice (11), we propose a model that RAR would be a negative regulator to allow transcription upon RA binding and that

PU.1 should instead be considered an authentic transactivator for *C/EBP*ε transcription that mediates the instructive role of PU.1 in granulocytic differentiation.

Elucidation of PML and PU.1 interaction during granulocytic differentiation. Although each PML isoform resides within discrete subnuclear compartments, there have been few reports on their innate biological activities in myeloid development. In this study, overexpression experiments were employed to delineate the function of each PML isoform. Because their protein expression levels could not be equalized, it might be possible that PML II, III, and V isoforms could not represent significant synergistic action with PU.1 simply due to their insufficient availability. In addition, since the expression level of transduced PML IV seems to be high compared to that of the endogenous one, the effect of PML IV on PU.1 action should be carefully interpreted. On the other hand, we think that PML overexpression employed in this study may mimic, at least in part, the increase of PML isoforms during the early stage of terminal granulocytic differentiation. We observed that PU.1 and PML mutually regulate each other. Although the mechanism remains unclear, we speculate that the increase of PML expression during RA-induced granulocytic differentiation might be due, at least in part, to an increase of PU.1 expression. Although the issue of isoform change during differentiation seems to be very important, we think at present that during granulocytic differentiation, PML (and PML IV) is regulated mainly quantitatively. In turn, PML IV specifically associates with PU.1 in vivo and enhances its function. Thus, PU.1 autoregulates its own transcriptional capability. The isoform-specific interaction was closely linked to the functional cooperation of PML and PU.1. Note that the ternary complex formation of PU.1/PML/p300 on the *C/EBP*ε promoter depends on PML recruitment and that it occurs rapidly after RA treatment, suggesting its role at the early stage of granulocytic terminal differentiation.

On the other hand, the relevance of the POD structure to transcriptional control remains elusive. Sumoylation of PML IV is a prerequisite for the normal architecture of PODs (39) and seems to be crucial for the transcriptional regulation of the PU.1/PML/p300 complex. Furthermore, the B boxes and coiled-coil domain of PML, essential for the formation of the normal POD structure, were required for the colocalization of PU.1 with PML IV. We also observed that PML VI cannot efficiently associate with PU.1 but does recruit it to PODs, and this activity is correlated with the cooperation of these proteins in the granulocytic differentiation of L-G cells, although PML VI does so less efficiently than PML IV. We speculate that PML VI indirectly regulates PU.1; e.g., PML VI may promote PML IV-mediated PU.1 targeting to PODs, although a complete understanding of the interaction between PML isoforms remains challenging. Another interesting finding is that PML IV augmented only the amount of C/EBPε expression but did not affect its time course profile in L-G cells expressing PU.1ΔPEST. Taken together with the finding that PU.1ΔPEST could not be efficiently recruited to PODs, we believe at present that PML IV-mediated ternary complex formation within the structurally integrated PODs would be required for the synergistic activation of transcription by the PU.1/PML/p300 ternary complex in vivo.

PU.1 and p300/CBP can directly interact, at least in vitro (33); however, we found that their association is rather weak compared with those of other transcription factors such as AML1b. We demonstrated that the PU.1/PML/p300 ternary complex is also formed on the target gene promoter and speculate that both PU.1 and p300 are efficiently assembled with the aid of PML IV, leading to the synergistic transcriptional activation of the target gene. As observed in LG/MT-PU.1 cells, efficient C/EBPε expression cannot be induced by PU.1 alone but requires the increase of PML expression and a possible reorganization of the PU.1 complex into an active form.

We also observed that PML IV increases PU.1 expression in both transient and stable coexpression systems throughout our experiments. Although its molecular mechanism remains to be elucidated, we speculate that the PML enhancement of PU.1 activity has an additional aspect of increased availability of PU.1 in addition to promoting the formation of the active transcription factor complex. Thus, PML IV seems to modulate PU.1 activity by both qualitative and quantitative mechanisms.

Reconsideration of the role of PML-RARA in APL: PML-RARA as a dominant-negative mutant causing dissociation of the PML-mediated transcription factor complex. Because RARA is a target for all APL-related chromosomal translocations, an alteration of its function must be required for promyelocytic transformation. PML-RARA has been thought to act as a dominant-negative inhibitor of the transactivator function of RARA. In addition, PML is a component of ligand-bound RARA complexes and regulates their activity (38). These observations have led to a proposed model in which PML-RARA acts as a dominant-negative inhibitor of the RA signaling pathway at multiple steps. On the other hand, since RARA has turned out to be dispensable for granulopoiesis, or rather acts as a transrepressor under physiological conditions in vivo (11), the enhanced repression of RA signals by PML-RARA would not likely be sufficient for fully elucidating the molecular mechanism of differentiation arrest in APL. It is not surprising, therefore, that the HDAC1-RARA fusion protein, a bona fide dominant-negative form of RARA, does not cause a block in myeloid differentiation in vivo and was not leukemogenic in those transgenic mice (18). Another study showed that homodimerizing artificial RAR fusions alone are poor initiators of leukemia, characterized by significant leukocytosis of mature neutrophils in vivo (28). Rather, the main role of the inhibitory effect of PML-RARA in RA signaling might only be priming for APL-like leukemia by the attenuation of spontaneous apoptosis (15). Therefore, the inhibitory roles of PML-RARA in normal PML function should be responsible for differentiation arrest.

We propose two different modes of action for PML-RARA inhibiting PML/transcription factor complexes (Fig. 8H). Because the association between PU.1 and p300 is weak, and largely depends on PML, PML-RARA heterodimerizes with PML and sequesters the PML/p300 complex from PU.1 (type I dominant-negative inhibition). On the other hand, AML1b still forms a stable complex with p300 regardless of the presence of PML I. In this case, PML-RARA gathers on the AML1b/p300 complex through heterodimerization with PML I and then attenuates the transcriptional activity, probably by recruiting corepressor complexes to overcome the histone acetyltransferase activity of p300/CBP (type II inhibition).

Thus, the inhibitory effects of PML-RARA would depend largely on the stability of a given transcription factor/p300 complex for PML.

Several lines of evidence suggest roles for C/EBPε in APL pathogenesis. Differentiation of BM cells from *C/EBPε*$^{-/-}$ mice is practically arrested at the promyelocyte stage, at least in vitro (34a). In addition, a previous excellent study shows that the overexpression of C/EBPε in APL rescues differentiation arrest in vitro as well as in vivo and prolongs the survival of mice transplanted with APL cells (29). On the other hand, repression of C/EBPε does not fully account for the pathophysiology of APL, because *C/EBPε*$^{-/-}$ mice do not capture the APL phenotype. Walter et al. previously reported that reduced PU.1 expression causes myeloid progenitor expansion and increased leukemia penetrance in mice expressing PML-RARA (30). Those authors also demonstrated that PML-RARA decreases the expression of PU.1 mRNA in PU.1-haploinsuficient mice by unknown mechanisms, causing the development of a hypomorphic PU.1 phenotype. Because PU.1 autoregulates its own expression (1), our results showing that PML-RARA inhibits the transcriptional capability of PU.1 agree with their findings and might partly explain the graded reduction of physiological PU.1 below a critical level, followed by the induction of myeloid leukemia (26). Thus, we suppose that the repression of PU.1 is one of the crucial mechanisms in PML-RARA leukemogenesis. Furthermore, PML-RARA is a multivalent suppressor for other C/EBP family members, including C/EBPα and C/EBPβ (4, 24, 29). We think that comprehensive inhibition of those transcription factors might be responsible for the full manifestation of APL.

There are four other types of APL-related chimeras that have been reported. Among them, NPM- or NuMA-RARA fusions do not affect the POD structure (8). In this respect, PML localization itself is not of primary importance for APL pathogenesis. On the other hand, disruption of the POD structure into microspeckles is invariably observed in t(15;17)-bearing APL, and the restoration of normal POD architecture is an early event in granulocytic differentiation following RA-induced degradation of PML-RARA (35). Therefore, POD structure-based PML function still seems to be a key target for the pathogenesis of PML-RARA-induced APL.

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