

In vivo analysis of the role of aberrant histone deacetylase recruitment and RAR α blockade in the pathogenesis of acute promyelocytic leukemia

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The promyelocytic leukemia–retinoic acid receptor α (PML–RAR α) protein of acute promyelocytic leukemia (APL) is oncogenic in vivo. It has been hypothesized that the ability of PML–RAR α to inhibit RAR α function through PML-dependent aberrant recruitment of histone deacetylases (HDACs) and chromatin remodeling is the key initiating event for leukemogenesis. To elucidate the role of HDAC in this process, we have generated HDAC1–RAR α fusion proteins and tested their activity and oncogenicity in vitro and in vivo in transgenic mice (TM). In parallel, we studied the in vivo leukemogenic potential of dominant negative (DN) and truncated RAR α mutants, as well as that of PML–RAR α mutants that are insensitive to retinoic acid. Surprisingly, although HDAC1–RAR α did act as a bona fide DN RAR α mutant in cellular in vitro and in cell culture, this fusion protein, as well as other DN RAR α mutants, did not cause a block in myeloid differentiation in vivo in TM and were not leukemogenic. Comparative analysis of these TM and of TM/PML^{−/−} and p53^{−/−} compound mutants lends support to a model by which the RAR α and PML blockade is necessary, but not sufficient, for leukemogenesis and the PML domain of the fusion protein provides unique functions that are required for leukemia initiation.

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Acute promyelocytic leukemia (APL) is characterized by nonrandom reciprocal translocations that always involve the *retinoic acid receptor α* (RAR α) gene on chromosome 17. RAR α fuses to the *promyelocytic leukemia* (PML) gene in the vast majority of APL cases (1, 2). These chromosomal translocations generate X–RAR α and RAR α –X fusion proteins. X–RAR α fusion proteins are oncogenic in vivo (2–6).

APL is characterized by a distinctive block of differentiation at the promyelocytic stage of myeloid development and by unique sensitivity to retinoic acid (RA) treatment (1, 2). RAR α binds to retinoic acid response elements (RARE) as a heterodimer with RXR α (1). In the absence of RA, the RAR α /RXR α heterodimer inhibits transcription through recruitment of histone deacetylases (HDACs; e.g., HDAC1), nuclear

receptor corepressors such as N-CoR or SMRT, and DNA methyltransferases (DNMT) (7). In the presence of a physiological concentration of RA (10^{−8} M), the RAR α /RXR α heterodimer dissociates from the HDAC complex and recruits transcriptional coactivators (8). In contrast, at physiological RA concentration, PML–RAR α protein acts as a dominant negative (DN) RAR α by forming aberrant complexes with HDAC and DNMT through the PML moiety of the fusion protein (4, 8–11). At a pharmacological dose of RA, PML–RAR α releases the HDAC complex and activates transcription, thus mimicking RAR α . Point mutations have been reported in the RAR α ligand-binding domain of PML–RAR α in cases with acquired resistance to RA (12). Collectively, these data suggest that aberrant recruitment of HDAC to RARE represents

a key event in APL leukemogenesis. However, the PML-RAR α oncoprotein can also interfere with the function of the remaining PML allele through heterodimerization (1, 2), and it remains to be determined to what extent each of these processes contributes to APL leukemogenesis.

RESULTS AND DISCUSSION

To determine whether aberrant HDAC-dependent transcriptional repression is necessary and sufficient for leukemogenesis, we generated transgenic mice harboring the following: (a) DN RAR α mutants along with their PML-RAR α counterpart and (b) an artificial HDAC-RAR α fusion protein along with its enzymatically inactive counterpart. We also studied in vivo an RAR α truncated mutant corresponding to the moiety of RAR α invariably shared by all the APL fusion proteins (1, 2) (Fig. 1 A).

RAR α E carries a glycine (G) to glutamate (E) substitution at amino acid 303 in the RAR α E domain that is responsible for ligand binding. This mutation leads to RA resistance and in vivo photocopies the RAR α KO phenotype (13). RAR α M₄ carries a leucine (L) to proline (P) substitution at amino acid 398 in domain E; and PML-RAR α M₄ harbors the same mutation found in RAR α M₄ (14). This mutation leads to RA-insensitive transcriptional repression (14).

HDAC1-RAR α expresses the full-length HDAC1 coding sequence fused to RAR α . HDAC1 is part of the aberrant PML-RAR α transcription (4, 9, 10). mHDAC1-RAR α carries a point mutation that abrogates HDAC1 enzymatic activity (histidine to phenylalanine at HDAC1 amino acid 199) (15). Δ RAR α carries a deletion that removes domain A from RAR α . This deletion is identical to the one observed in the X-RAR α fusion proteins and removes a domain responsible for transcriptional activation function (1, 16). These constructs were cloned in the *human cathepsin-G* (*hCG*) minigene (3, 4) and used to generate transgenic lines (Fig. 1, B and C).

We assessed whether HDAC1-RAR α displayed the distinctive features of the X-RAR α fusion proteins. We found that HDAC1-RAR α can homodimerize and heterodimerize with RXR α within the cell (Fig. 2, A and B). HDAC1-RAR α can effectively bind to the DR5 consensus sequence. Electromobility shift analysis (EMSA) produced a single HDAC1-RAR α protein DNA complex, whereas HDAC1-RAR α with RXR α formed two complexes (Fig. 2 C). These bands were abolished by the addition of an excess of unlabeled DR5 and super shifted with specific antibodies (Fig. 2 C). These data demonstrate that HDAC1-RAR α forms homo- and, and more efficiently, heterodimers that are able to bind to the DR5 consensus sequence, as previously demonstrated in the case of other APL fusion proteins (17, 18).

Next, we investigated whether HDAC1-RAR α acts as a transcriptional repressor. Vectors expressing RAR α , PML-RAR α , PLZF-RAR α , HDAC1-RAR α , mHDAC1-RAR α , and HDAC1 were transfected into 293T cells together with RAR β -luc, a luciferase reporter construct containing the RAR α -responsive promoter region of

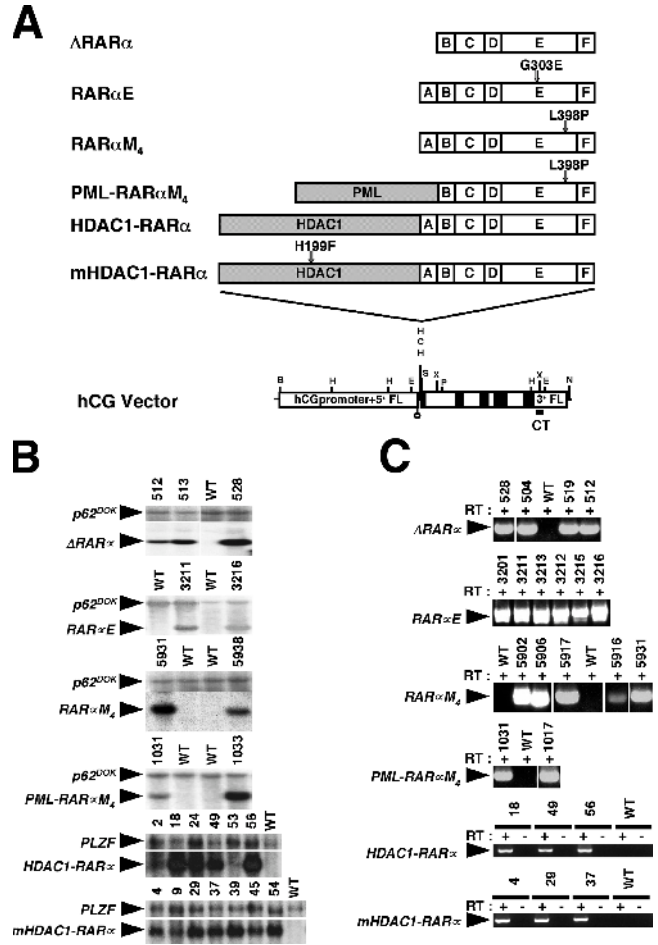


Figure 1. Generation of the mutant RAR α transgenic mice. (A) Mutant RAR α cDNAs were cloned into the Sall site of the *hCG* expression cassette. Shaded boxes: PML and HDAC1 sequences. Capital letters: RAR α domains. A schematic representation of the *hCG* is provided at the bottom of panel A. The regions flanking the 5' and 3' of the polylinker are indicated (5' FL and 3' FL, respectively). The 5' FL region comprises the *hCG* promoter. White boxes: exons. Restriction endonuclease sites are indicated. CT: probe for Southern blotting. (B) Southern blot of genomic DNA from transgenic founders digested with EcoRI and hybridized with probe CT. The transgene examined is indicated on the left side of the panel. Probes for the single copy genes *p62^{DOK}* or *PLZF* were used as internal standards. WT, wild type. The numbers above the individual panels indicate the founder lines. (C) RT-PCR analysis of RAR α mutant mRNA extracted from bone marrow cells. RT, reverse transcriptase.

RAR β . Luciferase assays demonstrated that HDAC1-RAR α acted as a potent transcriptional repressor (Fig. 3 A). As expected as the result of disruption of HDAC1 enzymatic activity, mHDAC1-RAR α showed a much weaker transcriptional repression. HDAC1-RAR α , PLZF-RAR α , and PML-RAR α repressed transcription equally well in the presence of RA, whereas mHDAC1-RAR α did not (Fig. 3 A). HDAC1-RAR α , therefore, acts as an aberrant transcriptional repressor and this property depends on the HDAC1 enzymatic activity.

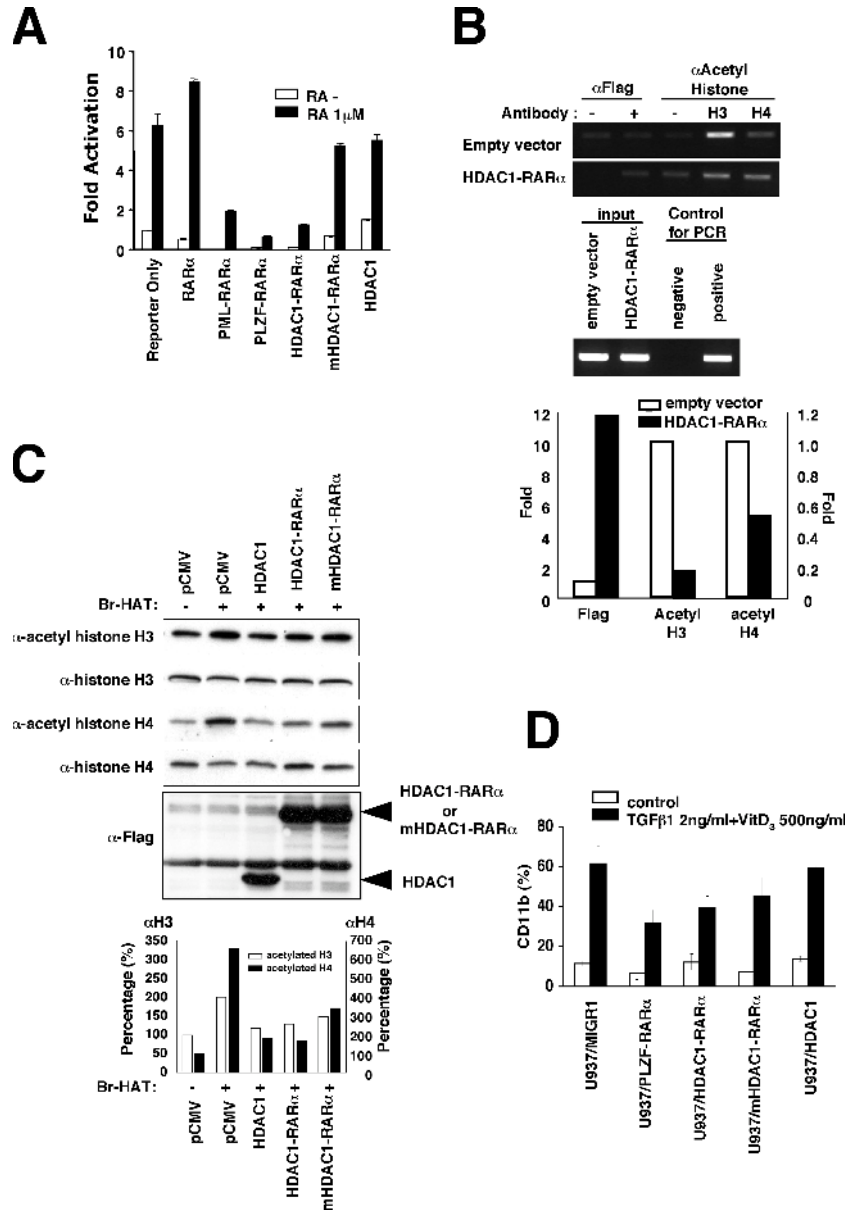


Figure 3. Biological properties of HDAC1-RAR α . (A) HDAC1-RAR α is a transcriptional repressor. Luciferase assay in transfected 293 cells. (black bars) RA-treated cells; (white bars) untreated cells. Luciferase activities were expressed relative to the value of lysates transfected with the reporter alone. (B) HDAC1-RAR α binds to RARE and deacetylates histone H3. (top) ChIP assay on lysate of transiently transfected 293T cells with the indicated antibodies. PCR was performed with RARE specific primers. (middle) PCR analysis performed with RARE-specific primers on the cell lysates was used for ChIP assay. (bottom) The intensity of the bands was determined by densitometry. The value obtained from the lysate transfected with the empty vector is expressed as 1. (C) HDAC1-RAR α represses the acetylation of histone H3 and H4 induced by BrHAT. 293T cells were cotransfected as indicated and the lysate was immunoblotted. The same membrane was

hybridized and stripped in series with the indicated antibodies. The arrows indicate transfected Flag-tagged proteins (bottom). The ratio of acetylated/total histone levels was assessed by densitometry and provided by the histogram at the bottom. The value obtained from the lysate transfected with pCMV alone is expressed arbitrarily as 100%. The value for acetylated H3/total H3 and acetylated H4/total H4 is provided. (D) HDAC1-RAR α inhibits the differentiation of U937. Cells were retrovirally transduced as indicated. Transduced cells were isolated by cell sorting and cultured with or without 2 ng/ml of TGF β 1 in addition to 500 ng/ml of vitamin D₃ for 96 h. Expression of CD11b was detected by flow cytometry. (black bars) percentage of treated cells expressing CD11b; (white bars) untreated cells. After treatment, CD11b expression is significantly reduced in PLZF-RAR α ($P = 0.01$) and HDAC1-RAR α ($P = 0.02$). Error bars indicate standard deviations.

such as U937 cells and interference with PML function seems not to be required for this function (20, 21). These observations support the notion that DN blockade of the

RAR α pathway is crucial for APL leukemogenesis. Our in vivo genetic analysis challenges this notion, allowing us to reach three major conclusions.

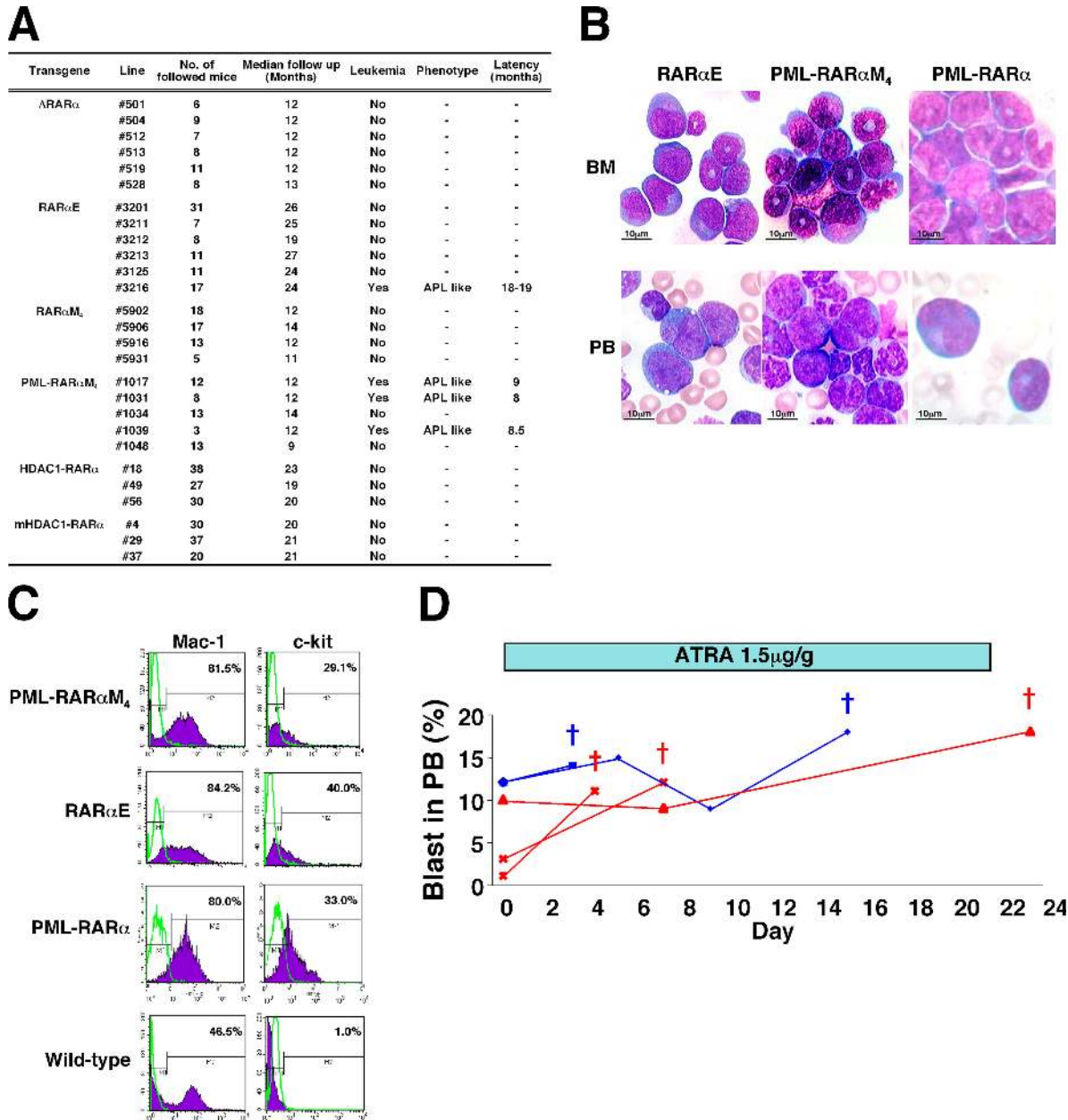


Figure 4. Characteristics of leukemias induced by *PML-RAR α M₄* and *RAR α E*. (A) Schematic representation of transgenic lines and respective leukemia incidence. (B) Peripheral blood (PB) and bone marrow cells (BM) from representative leukemic *RAR α E*, *PML-RAR α M₄*, and *PML-RAR α* transgenic mice stained with the Wright-Giemsa stain ($\times 1,000$). Note the presence of leukemic blasts in both BM and PB. (C) Flow cytometric analysis of BM cells from representative leukemic *RAR α E*, *PML-RAR α M₄*, and *PML-RAR α* transgenic mice. Anti-Mac-1 and c-kit antibodies were

used. (green line) Isotypic control. The percentages of positive cells are given in the respective histograms. (D) Leukemia induced by *RAR α E* is resistant to RA treatment in vivo. Percentage of leukemic cells present in the PB of two *RAR α E* transgenic mice (blue) and three nude mice (red) transplanted with leukemic cells obtained from *RAR α E* transgenic mice. The horizontal axis indicates the length of treatment in days. Crosses indicate the time of death of each animal. RA had no impact on the percentage of leukemic cells present in the PB.

The first major conclusion is that HDAC1-dependent DN blockade of *RAR α* function is neither sufficient to cause leukemia nor to block myeloid differentiation in vivo. The fact that only *PML-RAR α* and *PML-RAR α M₄* (which retain the X moiety), but none of the other DN *RAR α* mutants triggered leukemia in multiple transgenic lines dem-

onstrates that inhibition of *RAR α* per se is not sufficient to initiate leukemogenesis. Our experiments do not rule out that HDAC-chimeric constructs other than HDAC1-*RAR α* may display a leukemogenic effect. Indeed, corepressors do not solely recruit HDAC1, but also other types/classes of histone deacetylases, and *PML* interacts with both HDAC1

and 2. However, prior observations support this conclusion as *PLZF-RAR α* , transgenic mice develop leukemia, but not a block of myeloid differentiation, whereas *RAR α ^{-/-}* mice display a normal myeloid differentiation (4, 24).

The second major conclusion is that only one out of the six *RAR α E* lines developed APL after a long latency (1.5 yr) with very low incidence. This observation strongly suggests that blockade of *RAR α* function is necessary, but not sufficient, for leukemogenesis. Interestingly, these leukemias were resistant to RA, demonstrating that *RAR α E* functions in these leukemic cells as an RA-insensitive receptor.

The third major conclusion is that PML moiety is important in leukemogenesis not solely because it permits aberrant recruitment of HDAC1 and HDAC2, DNMT or homodimerization (11, 18, 25, 26) but also because it interferes with the tumor suppressive function of the wild-type PML gene product. Indeed, only PML-*RAR α* and PML-*RAR α M₄* lead to a DN disruption of the PML-NB both in vitro and in vivo (unpublished data). The critical role of PML functional inactivation is further underscored by the fact that APL is dramatically accelerated in *PML-RAR α /PML^{-/-}* mice (27). In addition, through the PML/X moiety, the fusion protein acquires aberrant gain-of-function properties (e.g., aberrant DNA binding activity) (28). Indeed, it has been shown that PML-*RAR α* homodimer binds specific DNA sites that are not preferentially recognized by the *RAR α /RXR α* heterodimer, thus suggesting the possibility that X-*RAR α* may exert oncogenic functions that are not derived from its DN activity against the *RAR α /RXR α* heterodimer (28, 29). This is supported by the fact that neither *RAR α M₄* nor *RAR α E* triggered leukemia even in the absence of PML: *MRP8-RAR α M₄/PML^{-/-}* and *MRP8-RAR α E/PML^{-/-}* mice did not develop leukemia during a 12-mo follow up (unpublished data and Kogan, S., personal communication). Interestingly, *RAR α M₄* did not trigger leukemia in the absence of *p53*, either; *MRP8-RAR α M₄/p53^{-/-}* compound mutants succumbed to lymphoma with incidence and onset similar to *p53* null mice (Kogan, S., personal communication).

We propose a model by which the combined inactivation of the X and *RAR α* pathways are both required, but not sufficient, for tumor initiation. PML-*RAR α* is bestowed with additional PML-dependent functional gains that critically contribute toward full-blown transformation. Nevertheless, additional genetic abnormalities are required for leukemogenesis even in the presence of the full-length oncogenic fusion protein, as strongly suggested by the long leukemia latency observed in any of the X-*RAR α* transgenic models and the recurrent chromosomal abnormalities that the leukemic blasts from these models invariably harbor at presentation (30, 31).

On the basis of this model, it remains to be explained why RA and HDAC inhibitors are effective in APL treatment. In this respect, it is tempting to speculate that the blockade of the *RAR α* pathway, while not sufficient for leukemia initiation, may be necessary for leukemia maintenance.

MATERIALS AND METHODS

Cells and expression vectors. Cells were obtained from the American Type Culture Collection. Vitamin D₃ was obtained from Sigma-Aldrich and TGF β 1 was obtained from PeproTech. Plasmids expressing *RAR α* , *RXR α* , *RAR α M₄*, *PML-RAR α* , *PML-RAR α M₄*, *PLZF-RAR α* , *HDAC1-FLAG* (provided by P.A. Marks and V. Richon, Memorial Sloan Kettering Cancer Center, New York, NY), His-BrHAT (provided by A. Tomita, Nagoya University, Nagoya Aichi, Japan), and *RAR α E* have been described previously (4, 13, 14, 19, 32). pSG5-HDAC1-*RAR α* carries the full-length HDAC1 gene fused in frame with the full-length *RAR α* . Mutant HDAC1-*RAR α* (mHDAC1-*RAR α*) was generated by site-directed mutagenesis. pSG5- Δ *RAR α* was generated by PCR. pCMV-PML-*RAR α* , pCMV-HDAC1-*RAR α* , pCMV-mHDAC1-*RAR α* , and pCMV-HDAC1 are pCMV-Tag 2B (FLAG-tagged) derivative (Stratagene). pCDNA3.1/His-HDAC1-*RAR α* , pCDNA3.1/His-RXR, pCDNA3.1/His-*RAR α E*, and pCDNA3.1/His-*RAR α M₄* were obtained by cloning the respective cDNAs into pCDNA3.1/His C (Invitrogen). To generate retroviral constructs, Flag-tagged *RAR α* , *PLZF-RAR α* , *HDAC1-RAR α* , mHDAC1-*RAR α* , and HDAC1 were cloned into pMIGR1. The sequence of each vector was confirmed sequencing.

Transgenic mice. *RAR α* mutants were cloned into the *Sall* site of the *hCG* minigene vector (3, 4). All constructs were sequenced. Egg injection was performed as described previously (3, 4). The mouse studies were approved and overseen by the Institutional Animal Care and Use Committee.

Antibodies, immunoprecipitations, and Western blot analyses. We used the antibodies specific for: *RAR α* (C-20) and *RXR α* (D-20) (Santa Cruz Biotechnology, Inc.), histone H3, H4, acetylated histone H3 and H4 (Upstate Biotechnology); PML (Chemicon International), M2 anti-Flag (Sigma-Aldrich), and anti-Xpress (Invitrogen).

Gel shift assay. *RAR α* , *RXR α* , and HDAC1-*RAR α* proteins were generated in vitro by TNT Coupled Reticulocyte Lysate Systems (Promega). Protein synthesis was confirmed by Western blot. Aliquots were used for gel shift analysis with the ³²P-labeled DR5 oligonucleotide: 5'-GGGACAAAG-GTCAACGAAAGGTCAGAGCCC-3' (29). For competition assays, we used 100-fold molar excess of unlabeled DR5. For supershift experiments, we used anti-*RAR α* , anti-*RXR α* antibodies, or normal rabbit IgG (Santa Cruz Biotechnology, Inc.).

Luciferase assay. 293T cells were cotransfected with *RAR β -luc* and pRL-TK (encoding firefly and renilla luciferases, respectively) and the relevant pSG5 expression constructs using Effectene Transfection Reagent (QIAGEN). Cultures were treated with 10⁻⁶ M of RA 24 h after transfection. Luciferase and *renilla* assays were done 48 h after transfection.

Chromatin immunoprecipitation (ChIP) assay. We used the ChIP assay kit (Upstate Biotechnology).

Retroviral transduction and flow cytometry analysis of U937 cells. Recombinant retroviruses were used to transduce U937 cells by spinoculation for three consecutive days. GFP-positive cells were sorted with MoFlo (DakoCytomation). Expression of mutant *RAR α* was confirmed by Western blot. CD11b was quantified by FACScan (BD Biosciences). These experiments were repeated five times. The unpaired Student's *t* test was used to compare CD11b expression between cells transduced with the MIGR1 vector and the ones transduced with MIGR1 vectors expressing *PLZF-RAR α* , *PML-RAR α* , *HDAC1-RAR α* , and mHDAC1-*RAR α* .

Southern blot analysis. Southern blots were done as described using probes for the *hCG*, *p62^{DOK1}* and *PLZF* genes (3, 4).

RT-nested PCR. Total RNA was extracted from mouse bone marrow with TRIzol (Invitrogen) and treated with DNase I. RT was performed using 2 μ g of total RNA with SuperScript First-Strand Synthesis System (Invitrogen). 1 μ l of cDNA was used for nested PCR.

Follow up of transgenic mice. Mice were monitored and diagnosed with leukemia as described previously (24, 27). Diagnosis was confirmed by morphological and flow cytometric analysis of bone marrow cells with Mac-1 (CD11b), Gr-1, c-kit (CD117), Sca-1, B220, CD3, and Ter119 antibodies (BD Biosciences).

Bone marrow transplants in nude mice and ATRA treatment. Leukemic cells were obtained from bone marrow and spleens of leukemic RAR α E TM. 4–8-wk-old Nu/J Hfh 11nu nude mice were injected with 2×10^6 leukemic cells intravenously. Transplanted nude mice (NM) were bled once a week. The leukemic TM and the NM that developed leukemia after transplantation received intraperitoneal injections of 1.5 μ g/g of RA daily (22).

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