**Promyelocytic Leukemia Zinc Finger-Retinoic Acid Receptor α (PLZF-RARα), an Oncogenic Transcriptional Repressor of Cyclin-dependent Kinase Inhibitor 1A (p21WAF/CDKN1A) and Tumor Protein p53 (TP53) Genes**

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**Background:** Promyelocytic leukemia zinc finger-retinoic acid receptor α (PLZF-RARα) is a transcriptional repressor generated by a chromosomal translocation between the PLZF and RARα genes in acute promyelocytic leukemia (APL-type) patients. The molecular interaction between PLZF-RARα and the histone deacetylase corepressor was proposed to be important in leukemogenesis. We found that PLZF-RARα can repress transcription of the p21WAF/CDKN1A gene, which encodes the negative cell cycle regulator p21 by binding to its proximal promoter Sp1-binding GC-boxes 3, 4, 5/6, a retinoic acid response element (RARE), and distal p53-responsive elements (p53REs). PLZF-RARα also acts as a competitive transcriptional repressor of p53, RARα, and Sp1. PLZF-RARα interacts with corepressors such as mSin3A, NCoR, and SMRT, thereby deacetylating histones Ac-H3 and Ac-H4 at the CDKN1A promoter. PLZF-RARα also interacts with the MBD3-NuRD complex, leading to epigenetic silencing of CDKN1A through DNA methylation. Furthermore, PLZF-RARα represses TP53 and increases p53 protein degradation by ubiquitination, further repressing p21 expression. Resultantly, PLZF-RARα promotes cell proliferation and significantly increases the number of cells in S-phase.

**Acute promyelocytic leukemia (APL)** is characterized by the clonal expansion of malignant myeloid cells that are blocked at the promyelocytic stage of hematopoietic differentiation. The RARα gene fuses to the PLZF gene at the t(11;17)(q23;q21) chromosomal translocation, leading to expression of a PLZF-RARα fusion protein that initiates APL. The PLZF-RARα fusion protein contains the entire N-terminal transcriptional effector regions and the first two zinc fingers of PLZF and all of RARα except the N-terminal A activation domain AF1. PLZF-RARα contains functional domains that are important to its protein functions, including transcriptional repression the pox-virus and zinc finger (POZ) domain of PLZF and the DNA binding domain of RARα. These structural features may explain the leukemogenic properties of this particular fusion protein. RARα binds to retinoic acid response elements (RAREs, direct repeats of (A/G)(G/T)TCA separated by 2 or 5 nucleotides), located in the promoters of many genes. RARα normally binds to RARE sites as a heterodimer with RXR. PLZF-RARα also binds to RAREs as a heterodimer with RXR (3, 4).

The PLZF-RARα oncoprotein functions as a transcriptional repressor in part by recruiting transcriptional corepressors and histone deacetylases (HDACs). However, the precise molecular mechanisms underlying the role of PLZF-RARα in oncogenesis and cell proliferation are poorly understood. It has been proposed that RARE-bound PLZF-RARα interacts with the NCoR/SMRT-HDAC complex to repress transcription, which appears to be a key pathogenic event in APL. Although the ligand/corepressor/coactivator binding domain of RARα alters its structure.

**Significance:** Oncoprotein PLZF-RARα represses transcription of the CDKN1A.
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ture upon binding to RA ligand, releasing a corepressor and recruiting a coactivator instead (5–7). PLZF-RARα does not release the corepressor–HDAC complex in the presence of RA, thus acting as a dominant-negative mutant form of RARα in APL (8). Accordingly, ATRA resistance of cells containing the PLZF-RARα fusion gene disrupts the RA signaling pathway that mediates myeloid differentiation, resulting in arrest at the immature promyelocytic stage (6, 9–12). Although some developmentally important PLZF and RARα target genes have been reported, the targets of PLZF-RARα that are important in cell proliferation and oncogenesis remain largely unknown but are presumed to be genes that contain a RARE in their promoters (e.g., CDKN1A). PLZF-RARα antagonizes RARE-containing genes normally up-regulated by RARα in the presence of retinoic acid. Thus, transcription of a battery of RARα target genes important in differentiation, development, and cell cycle arrest can be aberrantly repressed, leading to proliferation of the undifferentiated promyelocytes (2, 3).

p21, encoded by CDKN1A, inhibits the activity of the cyclin/cdk2 complex and is a major regulator of mammalian cell cycle arrest (13, 14). CDKN1A is primarily regulated at the transcriptional and translational levels (15). Whereas the induction of p21 predominantly leads to cell cycle arrest, the repression of CDKN1A expression may have a variety of outcomes, including cell proliferation, depending on the cellular context (15). The CDKN1A gene also is a transcriptional target of p53, which acts on the CDKN1A promoter distal p53 regulatory elements (14, 16) and plays a crucial role in mediating G1, G2, and S phase growth arrests upon exposure to DNA-damaging agents (15). In addition, Sp1 family transcription factors are major regulators that affect CDKN1A gene expression by binding to the proximal promoter (17). Recently, Krüppel-like transcription factors were also characterized as key regulators of CDKN1A expression that affect p53- and proximal Sp1-mediated regulation of CDKN1A transcription (18–24). p21 expression is activated by retinoic acid, and the CDKN1A promoter has a RARE with RARα interacts to activate transcription.

MBD3 (methyl-CpG-binding domain protein-3) is a component of the Mi-2/NuRD (Mi-2/nucleosome remodeling and deacetylase) chromatin remodeling complex that contains a nucleosome remodeling ATPase, HDAC1 and HDAC2 (histone deacetylases-1 and -2), and metastasis-associated protein 2 (MTA2) (25). MBD3, which has no intrinsic DNA binding activity, is targeted to methylated promoters through interactions with MBD2. At the promoter, MBD3 maintains transcriptionally repressed chromatin (26). Interestingly, the MBD3 protein was shown to be associated with the proximal promoter of CDKN1A in cancer cells and was released upon treatment of the cells with an HDAC inhibitor (27). However, the function and mechanism of MBD3 association with the CDKN1A promoter remains largely uncharacterized. By recruiting HDACs and DNA methyltransferases (DNMTs), MBD3 may act as an important transcriptional repressor of p21 during oncogenic transformation and cell proliferation (28).

Consequently, we investigated whether and how the CDKN1A gene encoding p21, a key regulator of cell cycle control and cell proliferation, is controlled by PLZF-RARα at the transcriptional level. Here, we show how various molecular interactions between PLZF-RARα, p53, Sp1, and MBD3 are all involved in regulation of CDKN1A. We found that the transcriptional regulation of CDKN1A by PLZF-RARα involves competitive binding of the transcription factors described above, modification of histones, and DNA methylation at the proximal CDKN1A promoter.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—The pSG5-PLZF-RARα plasmid was kindly provided by Dr. Jonathan D. Licht of Northwestern University (Chicago, IL). The CDKN1A-Luc plasmid was kindly provided by Dr. Yoshihiro Sowa, Kyoto Perpetual University of Medicine (Kyoto, Japan). The pGL2-CDKN1A-Luc, pGL2-TP53-Luc, pGL2-ARF-Luc, pGL2-MDM2-Luc, pcDNA3.1-p53, pcDNA3.1-Sp1, pG5–5x(GC-box)-Luc, and pGL2–6x(p53RE)–Luc and co-repressor expression vectors were either reported elsewhere or prepared by us (23).

Antibodies against p21, p53, HDAC1, HDAC3, MDM2, PLZF, RARα, Sp1, GAPDH, Myc tag, Ac-H3, Ac-H4, H3K4-Me3, H3K9-Me3, MBD3, HP1, MTA2, DNMT1, DNMT3b, mSin3A, NCoR, and SMRT were purchased from Upstate, Chemicon, Cell Signaling Technology, Abcam, Calbiochem, and Santa Cruz Biotechnology. Most of the chemical reagents, including TSA (trichostatin A), 5-aza-dC (5-aza-2'-deoxycytidine), and ATRA (all-trans-retinoic acid) were purchased from Sigma.

Cell Culture—HEK293, HL-60, HCT116 p53+/−, and HCT116 p53−/− cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HL-60 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS.

Ubiquitination Assays—H1299 cells grown in 10-cm dishes were co-transfected with 3 μg of pcDNA3-p53 and 2 μg of pcDNA3-His-ubiquitin in the presence or absence of pSG5-PLZF-RARα. Twenty-four hours after transfection, the cells were treated with 20 μM MG132 for 3 h and harvested. The cell pellets were resuspended in RIPA buffer (0.1% SDS, 1% Nonidet P-40, 1% EDTA, 50 mM, Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, complete Mini-Protease mixture (1 tablet/50 ml, Roche Applied Science)). 500 μg of the cell lysates was incubated with MagneHis™ nickel particles for 1 h at 4°C. After this step, the precipitated pellets were washed with buffer (0.5% Nonidet P-40, 200 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA) three times, resuspended in 2× SDS sample buffer, resolved by 10% SDS-PAGE, and analyzed by Western blot using an anti-p53 antibody.

Promoter DNA Methylation Analysis by Bisulfite DNA Sequencing—Genomic DNA was purified using the Wizard genomic DNA purification kit (Promega). Methylation analyses were performed by bisulfite conversion of genomic DNA using the EpiXplore™ Methyl Detection kit (Clontech). The primer sequences used to amplify the CDKN1A promoter region were sense, 5′-AGGGAGGAATGTGGTTTTGTA-3′, and antisense, 5′-AACAACCCACTCCACCTAAC-3′. The PCR product was cloned using the pGEM®-T Easy vector System I kit (Promega). Mini-scale plasmid DNA was prepared from more than 30 individually transformed Escherichia coli clones and sequenced.
Transcriptional Analysis of ARAF, MD2M, TP53, CDKN1A, and p53 Responsive Minimal Promoter—The pGL2-ARF-Luc, pGL2-MD2M-Luc, pGL2-TP53-Luc, pG13-Luc, pG5–5x(GC-box)-LUC, pGL2-CDKN1A-Luc promoter reporter fusion plasmids, and pSG5–PLZF-RARα were transfected into various combinations of cells (HEK293, HCT116 p53+/−, and HL-60) with Lipofectamine Plus reagent (Invitrogen). After 24–36 h of incubation, the cells were harvested and analyzed for luciferase activity. The reporter activity was normalized to either the total protein concentration or co-transfected β-galactosidase activity to correct for transfection efficiency.

Quantitative Real-time PCR (qPCR) of PLZF-RARα, MD2M, p53, GAPDH, and CDKN1A mRNA Expression in Cells—Total RNA was isolated from HEK293, HCT116 p53+/−, and HCT116 p53−/−, and HL-60 cells using TRIzol reagent (Invitrogen). cDNA was synthesized using 5 μg of total RNA, random hexamer (10 pmol), and SuperScript reverse transcriptase II (200 units) in a 20-μl reaction using a reverse transcription kit (Invitrogen). RT-qPCR was performed using SYBR Green master mix (Applied Biosystems). The following RT-qPCR oligonucleotide primers were used: PLZF-RARα forward, 5′-GAGACGTACGGGTGCGAGCTC-3′ and PLZF-RARα reverse, 5′-GAGACGTACGGGTGCGAGCTC-3′; MD2M forward, 5′-CCCCCTTAATGGCATTAGTAC-3′ and MD2M reverse, 5′-ACTGCTGACGGGCTTATTCCT-3′; p53 forward, 5′-CCTGAGTGGCTGCTGCTCAGAGTCTGGAAATC-3′ and p53 reverse, 5′-AAAGCTGTTCGCCCTCCAGTAGA-3′; p21 forward, 5′-AGGGGACAGCACAGAGGAAAG-3′ and p21 reverse, 5′-GGCTTTTTGAGTTGAGTGAATACTG-3′; GAPDH forward, 5′-ACCACAGTCCATGCATTCAAC-3′ and GAPDH reverse, 5′-TCCACACCCACCTGTGTCTGTA-3′.

Site-directed Mutagenesis of the CDKN1A Promoter—To investigate the role of each Sp1 binding site and RARα binding site, mutations were introduced into the p21 proximal promoter sequence using the QuikChange site-directed mutagenesis kit (Stratagene). The following oligonucleotides were used to introduce mutations into the core binding sequences of the GC-boxes and the RARE (only the top strands are shown): mSp1–1, 5′-CCCCGGGCACGCGGTTTCCGACGCGGCTCCGC-3′; mSp1–2, 5′-CCCCGGGCACGCGGTTTCCGACGCGGCTCCGC-3′; mSp1–3, 5′-CCCCGGGCACGCGGTTTCCGACGCGGCTCCGC-3′; mSp1–4, 5′-CCCCGGGCACGCGGTTTCCGACGCGGCTCCGC-3′; mSp1–5/6, 5′-TGGACGGCGCGGCGGCGTTTGGGTTTCTTATCAG-3′; mSp2–1, 5′-CCACAGCGAGAGGAGAAGAAAGGAGGAGAAG-3′; mSp2–2, 5′-CCACAGCGAGAGGAGAAGAAAGGAGGAGAAG-3′; mSp2–3, 5′-CCACAGCGAGAGGAGAAGAAAGGAGGAGAAG-3′; mSp2–4, 5′-CCACAGCGAGAGGAGAAGAAAGGAGGAGAAG-3′.

Immunoprecipitation Assays—HEK293, HCT116 p53+/−, and HL-60 cells (transfected with an expression vector, if necessary) were washed, pelleted, and resuspended in lysis buffer supplemented with protease inhibitors (20 μM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, complete Mini Protease mixture (1 tablet/50 ml, Roche Applied Science)). Cell lysates were pre-cleared, and the supernatants were incubated overnight with an anti-PLZF (or anti-p53, anti-Sp1, anti-RARα, anti-MBD3, anti-MTA2, anti-
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HP1, anti-DNMT1, anti-DNMT3b, or anti-GAPDH) antibody on a rotating platform at 4 °C and then incubated with protein A-Sepharose Fast Flow beads. The beads were collected, washed, and resuspended in equal volumes of 5 × SDS loading buffer. The immunoprecipitated proteins were separated using 8 and 10% SDS-PAGE. Western blots were performed with the appropriate antibodies as described above.

Oligonucleotide Pulldown Assays—HEK293, HCT116 p53+/−, HCT116 p53−/−, and HL-60 cells were lysed in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM DTT, and 0.5% Nonidet P-40). The cell extracts were then incubated with 1 μg of biotinylated double-stranded oligonucleotides (p53RE-1, p53RE-2, RARE, Sp1–1, Sp1–2, Sp1–3, Sp1–4, and Sp1–5/6) for 16 h. Oligonucleotide sequences were as follows (only the top strands are shown): Sp1–1, 5′-GATCGGGCGGCGGTCCCGG-3′; Sp1–2, 5′-GATCTCCCGGGCGGCGG-3′; Sp1–3, 5′-GATCCGAGCCGGGTCCCGGCTCT-3′; Sp1–4, 5′-GATCTTCGAGGCGGGGCTC-3′; Sp1–5, 5′-GATCGGGCGGCGGCGGTTCGCTC-3′; Sp1–6, 5′-GATCGGGCGGCGGCGGTTCGCTC-3′; and Sp1–5/6, 5′-GATCGGGCGGCGGCGGTTCGCTC-3′. To collect the DNA-bound proteins, the mixtures were then incubated with streptavidin-agarose beads for 2 h, washed with HKMG buffer, and the precipitates collected by centrifugation. The precipitates were then analyzed by Western blots using antibodies against PLZF, Sp1, p53, or GAPDH, as described above.

Flow Cytometry for Cell Cycle and Apoptosis Analysis—HEK293, HCT116 p53+/−, HCT116 p53−/−, and HL-60 cells were transfected with either a PLZF-RARα expression or control vector. Transfected cells were washed, fixed with methanol, and stained with a solution containing propidium iodide (50 μg/ml) and ribonuclease A (100 μg/ml) for 30 min at 37 °C in the dark. The DNA content, cell cycle profiles, and forward scatter of the cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer set to 488 (excitation) and 575 nm (peak emission). The data were analyzed using ModFit LT 2.0 (Verity Software House) and WindMDI 2.8 (Joseph Trotter, Scripps Research Institute).

To analyze the effect of PLZF-RARα on apoptosis, HEK293, HCT116, p53+/−, HCT116 p53−/−, and HL-60 cells were transfected with a PLZF-RARα expression vector or control vector. Transfected cells were washed, fixed with methanol, and stained with a solution containing propidium iodide (50 μg/ml) and ribonuclease A (100 μg/ml) for 30 min at 37 °C in the dark. The DNA content, cell cycle profiles, and forward scatter of the cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer set to 488 (excitation) and 575 nm (peak emission). The data were analyzed using ModFit LT 2.0 (Verity Software House) and WindMDI 2.8 (Joseph Trotter, Scripps Research Institute).

MTT Assays—Confluent HEK293, HCT116 p53+/−, HCT116 p53−/−, and HL-60 cells grown on 10-cm culture dishes were transfected with either a PLZF-RARα expression vector or control vector, transferred to 6-well culture dishes and grown for 0–4 days. At days 0, 1, 2, 3, and 4, the cells were incubated for 1 h at 37 °C with 20 μl of MTT/well (2 mg/ml). The precipitates were dissolved with 1 ml of dimethyl sulfoxide and the levels of cellular proliferation was determined by analyzing the conversion of MTT to formazan conversion using colorimetry at 540–600 nm.

RESULTS

PLZF-RARα Stimulates Cell Proliferation and Increases the Number of Cells in S-phase—We tested whether the oncoprotein PLZF-RARα can promote cell proliferation in HEK293, HCT116 cells, and eventually in HL-60 myeloid cells in the later
part of this study. Flow cytometry analysis of HEK293 and HCT116 cells transfected with a PLZF-RARα expression plasmid showed that PLZF-RARα stimulated cell cycle progression and increased the number of cells in S-phase (from 31.8 to 43.3%) (Fig. 1A). In agreement, MTT assays showed that PLZF-RARα significantly increased cell proliferation (Fig. 1B).

Because PLZF acts as a tumor suppressor with apoptotic activity in cells with a hematopoietic origin, we investigated whether PLZF-RARα induces apoptosis by analyzing HEK293 cells stained with Annexin V and propidium iodide. The cell populations undergoing early and late apoptosis were either minimal or negligible (from 1.11 to 3.75%) (Fig. 1A). RT-qPCR and Western blot (WB) analyses showed PLZF-RARα and endogenous MDM2, TP53, and CDKN1A expression in HEK293 cells transfected with a PLZF-RARα expression vector. GAPDH, control. D, structures of the four CDKN1A promoter constructs tested and transient transcription assays for CDKN1A gene expression. The PLZF-RARα expression vector and various CDKN1A promoter-luciferase fusion reporter plasmids (shown on the left) were transiently co-transfected into HEK293 cells and luciferase activity was measured. *, p < 0.05; t test.

PLZF-RARα Is a Transcriptional Repressor of the CDKN1A Gene Encoding p21—To understand how PLZF-RARα increases cell proliferation and exerts oncogenic properties, we investigated whether the oncoprotein PLZF-RARα could stimulate cell proliferation by controlling genes of the p53 pathway, important for cell cycle regulation. Transient transcription assays of HEK293 cells showed that PLZF-RARα repressed transcription of ARF, MDM2, TP53, and in particular, CDKN1A (Fig. 2A). RT-qPCR and Western blot analyses revealed that ectopic PLZF-RARα also repressed the expression of endogenous TP53, MDM2, and CDKN1A at the transcriptional level (Figs. 2B and 3C). Thus, PLZF-RARα regulates the upstream regulatory genes that eventually affect CDKN1A expression. We also examined which region of the CDKN1A promoter is important for transcriptional repression by PLZF-RARα in HEK293 cells. PLZF-RARα repressed the transcription of the four different CDKN1A promoters in a similar fashion, suggesting that PLZF-RARα may repress transcription by acting at the proximal promoter, which has six Sp1-binding GC-boxes (Fig. 2D).

PLZF-RARα Represses Transcription of the CDKN1A Gene by Binding to the Distal p53 Binding Elements and Decreasing p53 Stability and TP53 Transcription—Treatment with the DNA damaging agent etoposide increased CDKN1A expression by inducing p53 in HCT116 cells, which was repressed by PLZF-RARα (Fig. 3A). In HCT116 p53−/− cells, ectopic p53 expression increased CDKN1A expression, which was also repressed by PLZF-RARα (Fig. 3B). An additional transcriptional analysis using a pG5–6x(p53RE)-Luc construct with five copies of the distal p53 binding elements of the CDKN1A showed that PLZF-RARα blocked transcriptional activation of CDKN1A by p53 in Saos-2 cells (Fig. 3C). We observed a similar PLZF-RARα-mediated transcriptional repression of pG13-Luc, which contains 13 copies of the putative p53 binding element (Fig. 3D). Overall, our data suggest that PLZF-RARα can inhibit transcriptional activation of the CDKN1A gene by p53 at the p53 response element (p53RE) of the distal CDKN1A promoter.

We next analyzed whether ectopic PLZF-RARα affected p53 binding induced by etoposide in HCT116 cells. Although etoposide treatment did not affect the expression of PLZF-RARα significantly, transcriptional activation of TP53 and CDKN1A

**FIGURE 2. PLZF-RARα represses the transcription of p53 pathway genes in HEK293 cells.** A, transient transcription assays for the ARF, MDM2, TP53, and CDKN1A genes of the p53 pathway. The PLZF-RARα expression vector and promoter-luciferase fusion reporter plasmid were transiently co-transfected into HEK293 cells, and luciferase activity was measured. The results are the average of three independent assays. Bars, standard deviations. B and C, RT-qPCR and Western blot (WB) analyses showing PLZF-RARα and endogenous MDM2, TP53, and CDKN1A expression in HEK293 cells transfected with a PLZF-RARα expression vector. GAPDH, control. D, structures of the four CDKN1A promoter constructs tested and transient transcription assays for CDKN1A gene expression. The PLZF-RARα expression vector and various CDKN1A promoter-luciferase fusion reporter plasmids (shown on the left) were transiently co-transfected into HEK293 cells and luciferase activity was measured. *, p < 0.05; t test.
by etoposide was potently repressed by PLZF-RAR at both the mRNA and protein levels (Fig. 3, E–H).

PLZF-RARα also repressed transcriptional activation of CDKN1A by etoposide or ectopic p53 (Fig. 3). We also tested whether PLZF-RARα repressed transcription of CDKN1A in the absence of p53. Transient transcription assays in HCT116 p53−/− cells showed that PLZF-RARα could repress transcription of CDKN1A (Fig. 4A), and MTT assays of the same cells showed that PLZF-RARα significantly increased cell proliferation by 2.5-fold (Fig. 4B). Western blot and RT-qPCR analyses revealed that ectopic PLZF-RARα also repressed the expression of endogenous CDKN1A at both the protein and mRNA levels in HCT116 p53−/− cells (Fig. 4, C–F). These results suggest that transcriptional repression of CDKN1A by PLZF-RARα can be independent of p53.

Accordingly, PLZF-RARα may directly repress transcription of CDKN1A or indirectly, by repression of p53 activity or expression. Oligonucleotide pulldown assays showed that PLZF-RARα binds to and decreases p53 binding to p53REs (Fig. 5B). ChIP assays showed similar results in vivo (Fig. 5, C–G). Together, these results suggest that PLZF-RARα competes with p53 to bind to the two p53 binding elements and that this binding competition is important for transcriptional repression of CDKN1A. The transcription repression of TP53 by PLZF-RARα may also contribute indirectly to the repression of CDKN1A (Figs. 3, F and H; 5B, input lane 2, and 5I, input lane 2).

As protein-protein interactions between transcription factors can also repress transcription, we investigated whether PLZF-RARα directly interacts with p53. Co-immunoprecipita-
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Transcription repression of CDKN1A by PLZF-RARα can be independent of p53. A, transcription assays. HCT116 p53+/− and p53−/− cells were transiently co-transfected with a PLZF-RARα expression vector and a pGL2-CDKN1A-Luc (~2.3 kb) reporter plasmid, after which the cells were treated with etoposide and analyzed for luciferase activity. B, MTT assay of cell proliferation. HCT116 p53−/− cells transfected with either a pSG5 or pSG5-PLZF-RARα plasmid were grown for 1–4 days and analyzed for the MTT to formazan conversion using colorimetry at 540–600 nm. C–F, RT-qPCR and Western blot (WB) analyses showing PLZF-RARα and endogenous p53 and p21 expression in the HCT116 p53−/− cells transfected with the PLZF-RARα expression vector. GAPDH, control. *p < 0.05; N.S., not significant; t test.

PLZF-RARα Binds to the Proximal GC Boxes and Interacts with Sp1 to Repress the Transcription of CDKN1A—It has previously been shown that RARα-RXR complexes can activate transcription of CDKN1A by acting on the RARE in the CDKN1A distal promoter region (bp −1212 to −1194) (15, 30). In addition, the PLZF-RARα fusion protein retains a functional RARα DNA binding domain, and dysregulation of RARα target gene expression has been proposed to be an underlying cause of oncogenesis (5, 31). Accordingly, we tested whether PLZF-RARα could repress transcription of a CDKN1A-reporter fusion gene and the endogenous CDKN1A gene in HEK293 cells. Co-expression of RXR and RARα increased transcription and, in the presence of ATRA, further activated transcription. Regardless of the presence of ATRA, PLZF-RARα repressed both the CDKN1A reporter (Fig. 7A) and endogenous CDKN1A at their protein and mRNA levels (Fig. 7, B and C). Interestingly, we noticed that ATRA treatment increased p53 expression and PLZF-RARα decreased p53 expression, but ATRA did not affect PLZF-RARα expression (Fig. 7, B and C). Decrease in p53 expression by PLZF-RARα could further decrease CDKN1A expression.

Site-directed mutagenesis of the RARE of the CDKN1A promoter in the reporter plasmid and transient transcription assays revealed that the RARE is important for PLZF-RARα-mediated transcriptional repression because the mutation of
any of the bipartite RARE elements caused a loss of transcriptional repression (Fig. 7, D and E). Oligonucleotide pulldown assays indicated that PLZF-RARα/H9251 binds the RARE (Fig. 7F). Moreover, ChIP assays of PLZF-RARα/H9251 binding with both anti-PLZF and anti-HA antibodies revealed that ectopic PLZF-RARα binds to the RARE (Fig. 7G), resulting in CDKN1A transcriptional repression.

PLZF-RARα Epigenetically Silences the CDKN1A Proximal Promoter by Interacting with the MBD3-NuRD-HDAC3 Complex and DNMTs, Leading to DNA Methylation.

PLZF-RARα may inhibit transcription at the CDKN1A proximal promoter by interacting with the co-repressor-HDAC complex. ChIP assays showed that expression of ectopic PLZF-RARα decreased acetylation of histones H3 and H4 at the CDKN1A proximal promoter by 40–65% (Fig. 8C). In addition, epigenetic markers of transcriptional repression (H3K9-Me3) and activation (H3K4-Me3) were increased or decreased, respectively, by PLZF-RARα (Fig. 8D).

These data imply involvement of HDACs, DNMTs, and promoter DNA methylation in the transcriptional repression of CDKN1A by PLZF-RARα. Accordingly, we investigated whether the CDKN1A promoter region can be
methylated by Me-DIP (methylated DNA immunoprecipitation) assays. Ectopic PLZF-RARα increased methylation of the CDKN1A promoter region, as in the positive control AlphaX1 promoter, indicating that PLZF-RARα may repress transcription of CDKN1A through DNA methylation at the 15 CpGs of the proximal promoter region (bp, −139 to +30) (Fig. 8E). In control cells transfected with the pcDNA3 control construct, methylated bisulfite DNA sequencing showed that although some of the CpGs were methylated, only 1 of 20 CDKN1A promoter DNA strands sequenced was strongly methylated (13 of 15 CpGs), and only 2–3 moderately methylated promoter DNA strands were detected. In particular, the core CpG of Sp1 binding site-3, which is critical for transcriptional activation of CDKN1A, was methylated in 50% of the promoter DNA strands sequenced (17). In contrast, PLZF-RARα dramatically increased methylation at the CpG island of the CDKN1A proximal promoter, with virtually 70% (14 of 20) of the promoter DNA strands sequenced exhibiting extensively methylated CpGs (Fig. 8F).

Interestingly, all of the core CpGs of the six Sp1 binding GC-boxes were heavily methylated, which may inhibit promoter DNA binding and transcriptional regulation by Sp1 family and other Krüppel-like transcription factors.

Co-immunoprecipitation and Western blot analysis of either HEK293 cells or HEK293 cells transfected with a PLZF-RARα expression vector revealed that PLZF-RARα interacts with MBD3, the Mi-2-NuRD-HDAC3 complex, and the NuRD complex-associated DNMT1 and HP1 (Fig. 8G). ChIP analysis also showed that ectopic PLZF-RARα significantly increased the binding of MBD3, Mi-2-NuRD-HDAC3 complex (as monitored by diagnostic subunit MTA2), DNMT1/3b, and HP1 to the CDKN1A proximal promoter (Fig. 8H). PLZF-RARα, by interacting with MBD3, recruits the Mi-2-NuRD-HDAC3 complex and the complex-associated DNMT1/3b and HP1, likely resulting in CDKN1A promoter DNA methylation. Together, these results suggest that the CDKN1A promoter may be epigenetically silenced by histone deacetylation and DNA methylation.
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PLZF-RARα Stimulates Cell Proliferation and Represses CDKN1A Transcription in HL-60 Leukemia Cells through the Competitive Binding of p53, RARα, and Sp1, Histone Modifications, and DNA Methylation—We showed the molecular mechanism underlying the oncogenic properties of PLZF-RARα in HEK293 and HCT116 cells, and eventually tried to validate our findings in acute promyelocytic leukemia HL-60 cells. PLZF-RARα promoted cell proliferation of human HL-60 cells and repressed CDKN1A and TP53 expression (Fig. 9, A–C). ChIP assays also showed that ectopic PLZF-RARα decreased acetylation of histones H3 and H4 at the CDKN1A proximal promoter by 40–65% and increased or decreased histone methylation markers of repression (H3K9-Me3) or activation (H3K4-Me3), respectively (Fig. 9, D–F). Furthermore, Me-DIP assays showed that ectopic PLZF-RARα expression increased DNA methylation of the CDKN1A proximal promoter (Fig. 9G). These data imply that, as in HEK293 cells, PLZF-RARα represses CDKN1A transcription by HDAC and promoter DNA methylation in HL-60 cells.

ChIP assays of HL-60 cells transfected with a PLZF-RARα expression vector further revealed that ectopic PLZF-RARα increased binding of MBD3, the Mi-2-NuRD-HDAC3 complex (as monitored by MTA2 binding), DNMT1/3b, and HP1 to the CDKN1A promoter (Fig. 9, H–K). These results suggest that PLZF-RARα may repress CDKN1A expression epigenetically by histone deacetylation and/or methylation by recruiting the MBD3-Mi-2-NuRD-HDAC3 complex and its associated DNMT1/3b and HP1. These results show the molecular mechanisms we identified in HEK293 cells are also applicable to HL-60 human leukemia cells.

Epigenetic Repression of CDKN1A by PLZF-RARα in HL-60 Cells Can Be Partially Reversed by the HDAC Inhibitor TSA, the DNMT Inhibitor 5-Aza-2′-deoxycytidine, ATRA or Any Combination of these Three Drugs—PLZF-RARα can repress CDKN1A expression in HL-60 cells through epigenetic mechanisms that include histone deacetylation and promoter DNA methylation. Ectopic PLZF-RARα repressed CDKN1A through the deacetylation of histones H3 and H4 (Fig. 10, A and B). The ChIP data on markers of transcriptional activation and repression indicated that treating the cells with epigenetic derepressive agents (TSA and 5-aza-2′-deoxycytidine) combined with the RARα ligand ATRA did not completely derepress CDKN1A transcription to the level found in control cells (Fig. 10, H and J). Treating the cells with any of these agents alone or in combination did not affect PLZF-RARα (as judged by ChIP using an anti-PLZF antibody) binding or the control ChIP reactions (Fig. 9E). These results indicate that, of the several transcriptional repression mechanisms described above, CDKN1A transcriptional repression by the competitive binding of p53, PLZF-RARα, and Sp1 is quite significant. Because binding competition between these transcription factors is not likely affected by TSA and ATRA, the finding may explain why some APL patients with PLZF-RARα translocation are resistant to TSA and ATRA combination therapy and relapse.

FIGURE 7. PLZF-RARα represses the transcription of CDKN1A by binding to its RARE in vitro and in vivo. A, a transcription assay for the CDKN1A promoter in the presence of PLZF-RARα and ATRA or EtOH control in HEK293 cells. Cells were transiently co-transfected with the pGL2-CDKN1A-Luc WT (–2.3 kb), PLZF-RARα, and/or RXR-RARα expression vectors, treated with ATRA, and luciferase activity was measured. B, Western blot (WB) and RT-qPCR mRNA level analysis of the cell lysates transfected with control or PLZF-RARα expression vectors and treated with ATRA or EtOH control. C, a transcription assay for the CDKN1A proximal promoter (as monitored by MTA2 binding), DNMT1/3b, and HP1 in the presence of PLZF-RARα or ATRA in HEK293 cells. The cells were transfected with HA-PLZF-RARα expression vector, and luciferase activity was measured. F, oligonucleotide pulldown assays showing PLZF-RARα binding to the RARE. HEK293 cell extracts were incubated with biotinylated double-stranded oligonucleotide and analyzed, as described in the legend to Fig. 3 using an antibody against PLZF or IgG. G, qChIP assay showing PLZF-RARα binding at the RARE of the endogenous CDKN1A proximal promoter in HEK293 cells. The cells were transfected with HA-PLZF-RARα or PLZF-RARα expression vector. Antibodies against HA and PLZF and IgG were used in ChIP assays. The endogenous CDKN1A gene structure is shown. RAREs are indicated as RARs. The arrows indicate the binding positions of the qChIP oligonucleotide PCR primers flanking the RARE. Tsp (+1), transcription start site. *, p < 0.05; N.S., not significant; t test.
PLZF-RARα, a Transcriptional Repressor of CDKN1A and TP53

Although ATRA supplemented with the HDAC inhibitor TSA is effective in leukemia treatment, the addition of a DNMT inhibitor such as 5-aza-2′-deoxycytidine appears to be more effective in inhibiting the proliferation of HL-60 cells transfected with the PLZF-RARα expression vector (Fig. 10G). Because CDKN1A transcriptional repression caused by the competitive binding of p53, PLZF-RARα, RARα, and Sp1 and PLZF-RARα-mediated down-regulation of CDKN1A expression by PLZF-RARα (Fig. 10, H–J) persists in leukemic cells expressing the PLZF-RARα oncoprotein, a certain population of leukemic cells may still remain resistant to the three drug combination therapies. Accordingly, the fundamental goal for better treating RA-resistant APL leukemic patients may be to inactivate PLZF-RARα activity or block the expression of the fusion protein.

**DISCUSSION**

In APL-resistant ATRA treatment, the PLZF gene is fused with the RARα gene by chromosomal translocation (1, 2, 4). PLZF-RARα was presumed to antagonize the function of RARα by interfering with promyelocyte differentiation or/and disrupting myeloid-specific PLZF functions. PLZF-RARα promotes proliferation of promyelocytes (immature granulocytes) through the aberrant regulation of cell cycle-associated genes such as MYC (1–3, 32). However, the target genes and the mechanism by which the PLZF-RARα oncoprotein stimulates cell proliferation and blocks myeloid differentiation has remained largely unknown.

PLZF-RARα interacts with co-repressor-HDAC complexes such as NCoR/SMRT and Sin3A. PLZF-RARα contains two co-repressor binding sites, the CoR box of RARα and the POZ domain of PLZF. Retinoic acid releases HDAC complexes from the CoR box of PLZF-RARα, but not from the POZ domain, which accounts for the molecular basis of RA resistance in PLZF-RARα-type APL patients. Previously, an artificial minimal promoter system with a RARE was used to demonstrate that the histone deacetylase inhibitors TSA...
and ATRA can lift the transcriptional repression by PLZF-RARα and synergistically activate reporter gene expression in CV-1, NB4, and U937 cells (5, 33). That study provided a basis for the effective growth suppression of ATRA-resistant cells in CV-1, NB4, and U937 cells (34), the true targets (p53, Sp1, RAR and ATRA) of the oncoprotein PLZF-RARα. These include CDKN1A, which is repressed by PLZF-RARα binding (34). The proximal GC-boxes and distal p53REs that are critical for basal transcription help PLZF-RARα to repress expression of CDKN1A proximal promoter in HL-60 cells transfected with a PLZF-RARα expression vector. Alpha X1, positive control. H–K, qChIP assays of MBD3, NuRD (MTA2), DNMT1 and -3b, and HP1 binding at the proximal Ac-H3, Ac-H4, H3K4-Me3, and H3K9-Me3 levels at the indicated regions (Fig. 8A) using the indicated antibodies. G, Me-DIP assays to assess DNA methylation of the endogenous CDKN1A proximal promoter in HL-60 cells transfected with PLZF-RARα expression vector. Alpha X1, positive control. N.C., negative control; P.C., positive control; N.S., not significant; t test.

Consequently, we propose a hypothetical model for the transcriptional regulation of CDKN1A by PLZF-RARα (Fig. 11). Under normal cellular conditions in which p53 levels are low and no PLZF-RARα is present, p21 is expressed at a low level and cells proliferate normally. When cells are challenged with genotoxic stress, however, the tumor suppressor p53 is markedly induced and activates CDKN1A transcription by interacting with its p53REs and Sp1 bound at the Sp1-binding GC-box 3. The induced p21 protein then stops progression of the cell cycle and allows the cells to either repair DNA damage or undergo apoptosis. When cells express PLZF-RARα following chromosomal translocation, PLZF-RARα represses CDKN1A transcription by binding to proximal GC-boxes 3, 4, and 5/6, RARE, and the distal p53REs that confer the ability of PLZF-RARα to repress expression of CDKN1A proximal promoter in HL-60 cells transfected with PLZF-RARα expression vector. Alpha X1, positive control. H–K, qChIP assays of MBD3, NuRD (MTA2), DNMT1 and -3b, and HP1 binding at the proximal CDKN1A promoter in HL-60 cells transfected with a PLZF-RARα expression vector. Alpha X1, positive control. N.C., negative control; P.C., positive control; N.S., not significant; t test.
FIGURE 10. Proliferation of HL-60 cells is increased by ectopic PLZF-RARα and decreased by TSA, 5-aza-2'-deoxycytidine, ATRA, or any combination thereof. Transcriptional repression of CDKN1A by PLZF-RARα is derepressed by the reagents. A–D, qChIP assays of histone modifications at the endogenous CDKN1A gene proximal promoter using antibodies against IgG, Ac-H3, Ac-H4, H3K4-Me3, and H3K9-Me3. Cells were transfected with a PLZF-RARα expression vector and the cell lysates were immunoprecipitated with the indicated antibodies. E and F, qChIP-PCR assays of PLZF-RARα binding at the endogenous CDKN1A proximal promoter. Cells were transfected with a PLZF-RARα expression vector and the cell lysates were immunoprecipitated with an anti-PLZF antibody. Control ChIP assays using IgG are shown at the right (F). G, MTT assay of HL-60 cell proliferation. The cells were transfected with the pSG5-PLZF-RARα plasmid, treated with 200 nM TSA, 4 μM 5-aza-2'-deoxycytidine, 2 μM ATRA for 12 h or any combination thereof, grown 1–4 days, and analyzed for the MTT to formazan conversion using colorimetry at 540–600 nm. H, Western blot (WB) analysis of ectopic PLZF-RARα expression in HL-60 cells treated with various combinations of reagents. I and J, RT-qPCR analysis of PLZF-RARα and p21 mRNA. HL-60 cells transfected with the PLZF-RARα expression vector were further treated with the reagents and analyzed for expression of ectopic PLZF-RARα and endogenous p21 mRNA. *, p < 0.05; N.S., not significant; t test.
Furthermore, PLZF-RARα potently represses transcription of TP53 and decreases p53 stability by inhibiting p53 acetylation and increasing p53 ubiquitination. p53 is an upstream transcriptional activator of CDKN1A. PLZF-RARα blocks the induction of CDKN1A by repressing the expression of de novo p53 and promoting the degradation of p53 through decreased p53 acetylation and increased p53 ubiquitination (Figs. 5, B, G, I, and J; and 11, C and D). PLZF-RARα significantly affects not only the transcription of CDKN1A, but may also affect other p53 and RARα target genes important for apoptosis, differentiation, cell cycle regulation, etc.

However, the transcriptional repression of CDKN1A mediated by PLZF-RARα appears to be more complex than the repression by competitive binding among transcription factors and HDAC activity, and additionally involves epigenetic silencing by histone deacetylation and DNA methylation. Interestingly, the methylated DNA-binding protein MBD3, which is one of the subunits of the Mi-2-NuRD-HDAC3 complex, was found to be associated with the proximal promoter of CDKN1A (35). PLZF-RARα interacts with MBD3 and recruits the Mi-2-NuRD-HDAC3 complex and the NuRD-associated DNMT1/3b and HP1, which eventually leads to CDKN1A promoter DNA methylation.

Using HEK293 and HCT116 cells, we were able to consistently show that PLZF-RARα has proto-oncoprotein characteristics with the capacity to transform cells and stimulate cell proliferation by repressing CDKN1A expression. However, one might argue that the molecular mechanism of transcriptional regulation of CDKN1A by PLZF-RARα revealed in HEK293 and HCT116 cells might not be true in leukemic cells, which is often true depending on the transcription factors and cellular contexts. We were able to demonstrate that PLZF-RARα also represses transcription of CDKN1A in human promyelocytic leukemia HL-60 cells.

The PML-RARα fusion protein has an abnormally high affinity for corepressors, and the protein switches to an activator by
releasing corepressors at pharmacological doses of ATRA (5, 36). However, the PLZF-RARα fusion protein is ATRA-resistant and does not release corepressors. Previously, HDAC inhibitors such as TSA and butyrate were shown to block PLZF-RARα-mediated repression of reporter genes (5), and the combination of ATRA and the HDAC inhibitor suberoyl anilide hydroxamic acid (SAHA) was reported to be sufficient for clearing leukemic blasts from the peripheral blood of mice harboring PLZF-RARα (12). Our study suggests that DNMT inhibitors such as 5-aza-2′-deoxycytidine in combination with ATRA and HDAC inhibitors may be more effective in derepression of the PLZF-RARα target genes involved in cell differentiation, cell proliferation, and oncogenesis. Thus, PLZF-RARα-type APL patients may be more effectively treated by the addition of DNMT inhibitors to the ATRA plus HDAC inhibitor regimen. However, because the repression of CDKN1A is in part, due to competitive binding between p53, PLZF-RARα, RARα, and Sp1, and the fact that transcription repression of TP53 by PLZF-RARα persists in leukemic cells with PLZF-RARα, a certain population of leukemic cells may still remain resistant to the above described three drug combination therapy. Thus, an improved treatment of ATRA-resistant APL patients could be to inactivate PLZF-RARα activity or block the expression of the fusion protein.

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