

Role of the Polycomb Repressive Complex 2 in Acute Promyelocytic Leukemia

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SUMMARY

Epigenetic changes are common alterations in cancer cells. Here, we have investigated the role of Polycomb group proteins in the establishment and maintenance of the aberrant silencing of tumor suppressor genes during transformation induced by the leukemia-associated PML-RAR α fusion protein. We show that in leukemic cells knockdown of SUZ12, a key component of Polycomb repressive complex 2 (PRC2), reverts not only histone modification but also induces DNA demethylation of PML-RAR α target genes. This results in promoter reactivation and granulocytic differentiation. Importantly, the epigenetic alterations caused by PML-RAR α can be reverted by retinoic acid treatment of primary blasts from leukemic patients. Our results demonstrate that the direct targeting of Polycomb group proteins by an oncogene plays a key role during carcinogenesis.

INTRODUCTION

The Polycomb group (PcG) of proteins catalyze the addition of a methyl group at lysine 27 of histone H3 (H3K27me) (Margueron et al., 2005). Together with DNA methyltransferases (Feinberg and Tycko, 2004; Jaenisch and Bird, 2003; Jones and Baylin, 2002), which are responsible for methylation at cytosine bases within CpG dinucleotides, PcG-proteins control the transcriptional program and preserve the identity of each cell type, thus playing a central role in the maintenance of epigenetic memory throughout the lifetime of organisms. Perturbation of the epigenetic patterns at developmentally and cell-cycle regulated genes is a hallmark of cancer cells, and overexpression of PcG proteins (e.g., BMI1 or EZH2, reviewed Lund and van Lohuizen, 2004a) and

DNA methyltransferases (DNMTs) has been documented in human tumors (Jones and Laird, 1999). Polycomb group (PcG) of proteins was first identified in *Drosophila* as transcriptional repressors necessary for the temporal and spatial regulation of homeotic genes during development (Francis and Kingston, 2001). They are conserved throughout evolution and exist in at least two separate protein complexes both in *Drosophila* and in mammals: the Polycomb repressive complex 1 (PRC1) and the Polycomb repressive complex 2 through PRC4 (PRC2/3/4). PRC1 is a large complex including several proteins such as BMI1, HPH1-3, HPC proteins, and RING1-2 (Lund and van Lohuizen, 2004b; Ringrose and Paro, 2004). The core components of PRC2/3/4 are EZH2, different isoforms of EED, SUZ12, and the histone-binding proteins RbAp48/46 (Zhang and Reinberg, 2001).

SIGNIFICANCE

Polycomb repressive complex 2 has been strongly implicated in cancer development, but to date, mechanistic insight into the function of PRC2 in cancer cells is lacking. In addition, in mammalian cells, it is not well understood how PRC2 is targeted to promoter regions. Using as paradigm the oncogenic transcription factor PML-RAR α , we provide evidence of a direct recruitment of PRC2 to tumor suppressor genes during the initial steps of leukemogenesis. Reversion of PRC2-mediated silencing restores cell differentiation, thus identifying a potential avenue for therapeutic intervention.

Polycomb-mediated gene silencing is initiated by methylation of H3K27 by Ezh2, a component of PRC2 complex. Successively, binding of HPC proteins leads to recruitment of the PRC1 which is thought to be required for the maintenance of repression. It has been shown that in *Drosophila*, the PRC2 complex is targeted to genes by the recognition of specific sequences called Polycomb repressive elements (PREs). However, the homologous sequences in mammals remain elusive and the mechanism of Polycomb recruitment is unclear.

The oncogenic fusion protein PML-RAR α , generated by the chromosomal translocation of the PML gene on chromosome 15 and the retinoic receptor α (RAR α) on chromosome 17, is known to be responsible for 99% of acute promyelocytic leukemia (APL) cases (Di Croce, 2005). In absence of retinoic acid (RA), RAR α interacts with corepressor complexes and represses transcription of its target genes. The conformational change caused by the binding of physiological concentration of RA triggers the dissociation of the corepressors, promoting the recruitment of activators, such as histone acetylases, and the activation of transcription. Due to its capability to form oligomers (Licht, 2006), PML-RAR α acts as a constitutive repressor becoming insensitive to physiological concentrations of RA. However, pharmacological doses of RA can lead to partial derepression of PML-RAR α target genes (Pandolfi, 2001).

Many open questions remain, such as whether PcG repressor complexes are recruited to specific promoters by oncogenic transcription factors, similar to those observed for DNMTs, and, if so, whether these two main epigenetic memory systems are concomitantly involved in the establishment and maintenance of aberrant gene silencing during tumorigenesis.

RESULTS

PML-RAR α Interacts with Polycomb Repressive Complex

We have previously shown that the oncogenic protein PML-RAR α , found in 99% of acute promyelocytic leukemia (APL), recruits several chromatin modifiers, including DNMTs (Di Croce et al., 2002), to its target genes. In a separate line of investigation, we recently demonstrated that the PRC2 complex associates with DNMT1 and DNMT3a/b (Vire et al., 2006). To investigate whether PML-RAR α associates with physiological levels of the PRC2/3/4 complexes, we performed coimmunoprecipitation experiments using lysates from the two best-characterized APL model systems: patient-derived NB4 and U937-PR9 cells, which are hematopoietic precursor cells carrying the PML-RAR α coding sequence under the control of a zinc-inducible promoter (Di Croce et al., 2002; Grignani et al., 1998; Lin et al., 1998).

Immunoblot analysis of anti-PML-RAR α immunoprecipitates revealed the existence of endogenous complexes of PML-RAR α with SUZ12, EZH2, and EED (Figures 1A and 1B). Furthermore, we transiently transfected 293T cells with expression vectors for Flag-tagged

PML-RAR α and the trimeric PRC2 complex (EED/EZH2/SUZ12) (Cao and Zhang, 2004a; Pasini et al., 2004). We found that all PRC2 components could be specifically immunoprecipitated with PML-RAR α , using the anti-Flag antibody (Figure 1C). Similar results were obtained performing the reverse coimmunoprecipitation experiments, using antisera specific for HA-EZH2 or SUZ12, and an anti-RAR α antibody for visualization of the immunoprecipitated complexes (Figures S1A and S1B). Interestingly, the presence of SUZ12 is required for the PML-RAR/EZH2 interaction and for the enzymatic activity of the associated EZH2 (see below), as demonstrated by immunodepletion (Figure S3C) and interference experiments (Figure S3D).

To identify the PML-RAR α moiety involved in the interaction with PRC components, we transiently transfected 293T cell with an expression vector with either wild-type RAR α or PML-RAR α . Coimmunoprecipitation experiments suggested that PML-RAR α has a greater affinity for EZH2 than does RAR α (Figure 1D). As PML-RAR α forms oligomers (Minucci et al., 2000), we next investigated whether oligomerization of RAR α could enhance its interaction with EZH2. We thus compared PML-RAR α with p53-RAR α , which was generated by fusing RAR α to the tetramerization domain present in human p53 (amino acids 324–355). Our result suggests that RAR α -forced oligomerization is sufficient to attract EZH2-containing complexes, albeit less efficiently than the oncoprotein PML-RAR α (Figure 1D). Finally, using a similar approach, we demonstrated that PML also interacts with EZH2 (Figure 1E) as reported for other corepressors (Di Croce et al., 2002; Khan et al., 2001).

We next tested whether, in addition to contributions by the PML moiety and the oligomerization status of RAR α , the presence of the NCoR/SMRT/HDAC3 corepressor complex (Guenther et al., 2000; Villa et al., 2006) is also required for the PML-RAR α interaction with EZH2. We used the AHT mutant, which contains a triple mutation that reduces the ability of PML-RAR α to recruit HDAC/NCoR complex through its RAR α moiety (Grignani et al., 1998; Lin et al., 1998). We found that the PML-RAR α AHT mutant retains the capability to interact with EZH2 (Figure S1C).

The above results prompted us to investigate whether RAR α associates with PRC2/3/4 complexes in vivo, and whether PcG can bind to RARE-containing promoter. We took advantage of U937-PR9 cells, in which only wild-type RAR α is present in the cell nucleus in the absence of Zn²⁺, while upon Zn²⁺ induction, both PML-RAR α and RAR α are expressed. Western blot analysis of SUZ12-immunoprecipitated complex revealed that only PML-RAR α , and not endogenous RAR α , was coimmunoprecipitated (Figure 1B, right panel). Results from reverse experiments confirmed that EZH2 specifically associates only with the oncoprotein and not with endogenous wild-type RAR α (Figure 1B, left panel). Finally, no interaction between PcG proteins and endogenous RAR α was observed in HL60 cells, which are derived from an AML patient and are negative for PML-RAR α but positive for wild-type RAR α (Figure S1D).

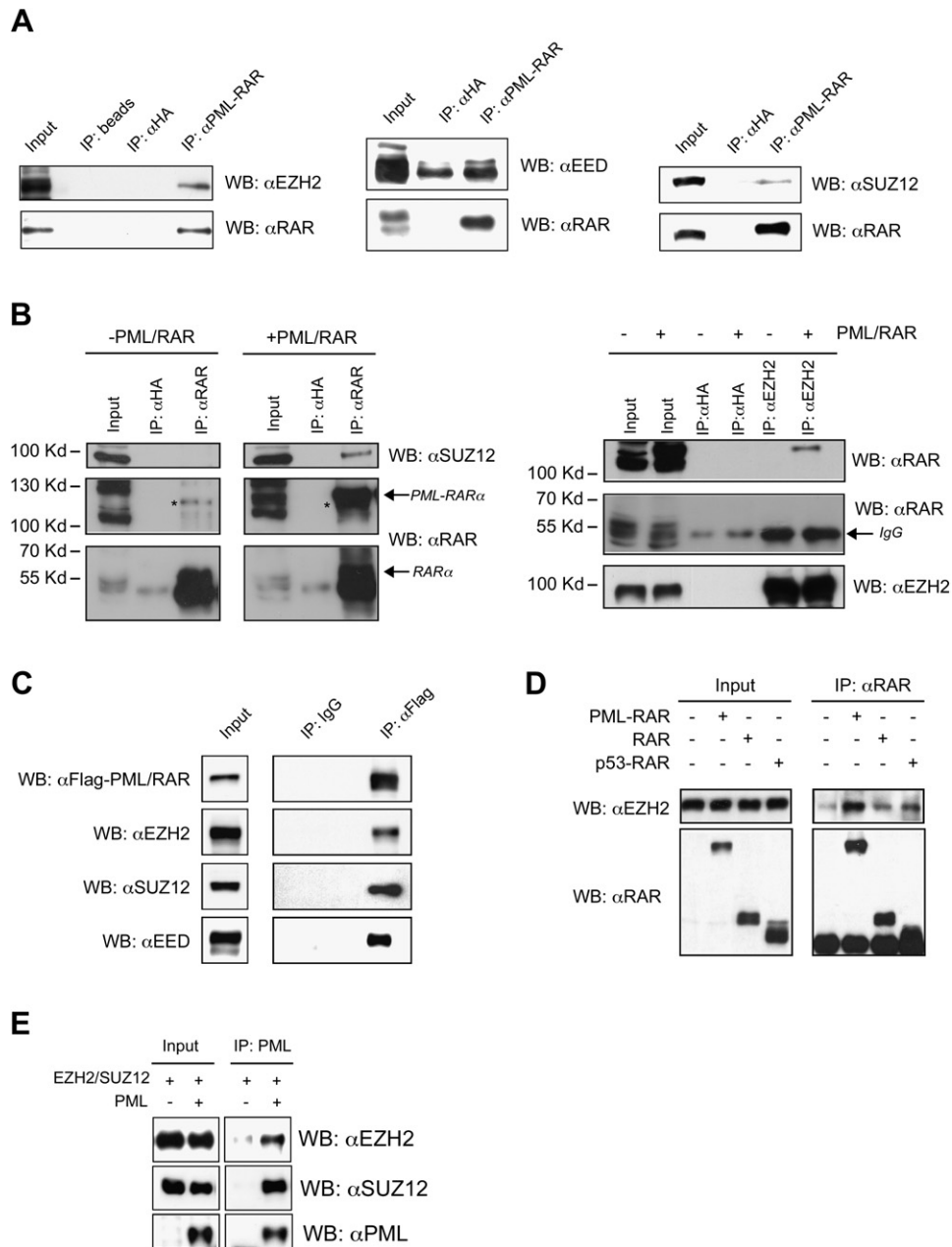


Figure 1. PML-RAR Specifically Interacts with the PRC2 Complex

(A) Endogenous interaction between PML-RAR α and the PcG complex. Cell extract from NB4 cells was immunoprecipitated with the PGM3 antibody. Western blots of input lysate or of immunoprecipitates were analyzed using antisera against RAR α , EZH2, EED, or SUZ12.

(B) Interaction between endogenous PcG and PML-RAR α or RAR α . Cell extract from U937-PR9 untreated or treated with 100 μ M Zn was immunoprecipitated with an antibody against RAR α or EZH2. Western blots of input lysate or of immunoprecipitates were analyzed using antisera against RAR α , Σ YZ12, or EZH2. Asterisks indicate nonspecific bands.

(C) Interaction between PML-RAR α and PRC2 complex. 293T cells were transfected with Flag-PML-RAR α , EZH2, SUZ12, and EED expression vectors, and extracts were immunoprecipitated with an antibody against Flag. Western blots of input lysate or immunoprecipitates were analyzed using antisera as indicated in the figure.

(D) Interaction between EZH2 and PML-RAR α , RAR α , or p53-RAR α . 293T cells were transfected with PML-RAR α , RAR α , or p53-RAR α expression vectors, and extracts were immunoprecipitated with an antibody against RAR α . Western blots of input lysate or of immunoprecipitates were analyzed by using antisera as indicated in the figure.

(E) Interaction between EZH2 or SUZ12 and PML. 293T cells were transfected with PML and EZH2 or SUZ12 expression vectors, and extracts were immunoprecipitated with an antibody against PML. Western blots of input lysate or of immunoprecipitates were analyzed by using antisera against PML, EZH2, or SUZ12.

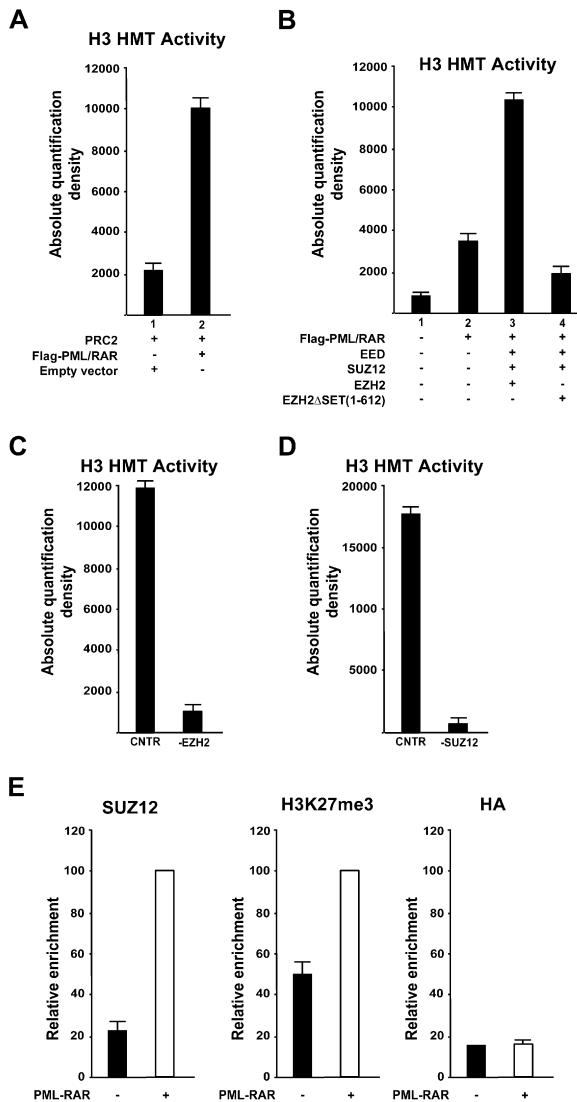


Figure 2. PML-RAR α Recruits Enzymatically Active PRC2 Complex to Its Target Genes

(A) Substrate preference of the PRC2 complex associated with PML-RAR α . Flag-PML-RAR α was purified at high-salt concentration (300 mM NaCl) to minimize possible interaction with endogenous proteins (see also Figure S2B), and incubated with baculovirus-expressed PRC2 complex (see also Figure S2A). After pull-down using antisera against Flag, the immunoprecipitated material was incubated with oligonucleosomes and assayed for HMT activity. Quantification of the HMT activity is shown.

(B) The EZH2 SET domain is required for PML-RAR α associated H3 HMT activity. 293T cells were transfected with Flag-PML-RAR α , EZH2, EZH2 Δ SET, SUZ12, or EED expression vectors, as indicated in the figure. After immunoprecipitation with an antisera against Flag, HMT assay was performed as in Figure 2A.

(C and D) Immunodepletion of the PRC2 complex abrogates the histone H3 HMT activity associated with PML-RAR α . Lysate of 293T cells expressing Flag-PML-RAR α and the PRC2 complex was immunodepleted using mouse anti-EZH2 or anti-SUZ12 antibody. Flow-through fractions were collected and used for PML-RAR α -associated HMT activity assay as in Figure 2A.

(E) PML-RAR α recruits the PcG complex to the RAR β 2 promoter region. U937-PR9 cells, treated sequentially with RA (1 nM for 24 hr)

PML-RAR α Recruits Active PRC2 Complex to Target Genes

To test if the PRC2 complex interacting with PML-RAR α is enzymatically active, we performed an in vitro histone methyltransferase assay (HMT) (Cao and Zhang, 2004a; Pasini et al., 2004) with the purified PML-RAR α and PRC2 complex (Figure 2A and Figure S2A). First, baculovirus-purified PRC2 components were coprecipitated with 293T-purified FLAG-PML-RAR α and extensively washed (Figure S2A). Note that Flag-PML-RAR α was purified in high salt extraction buffer in order to prevent interaction with endogenous HMT activities (Figure S2B). The bound complex was incubated in presence of oligonucleosomes and 3 H-SAM, and methylation of histones was measured by radiography (Figure 2A and Figure S2A). Indeed, the PML-RAR α /PRC2 complex was enzymatically active specifically on histone H3, likely on lysine 27 (Cao and Zhang, 2004a; Pasini et al., 2004). To support these in vitro results, we performed the HMT assay on the PRC2 complex, which coimmunopurified with ectopically expressed PML-RAR α in 293T cells (Figure 2B and Figure S3A). A strong HMT activity specific for histone H3 was visible when both PML-RAR α and PRC2 were cotransfected (Figure 2B and Figure S3A, lane 3). In cells expressing Flag-PML-RAR α alone (lane 2), the observed HMT activity is due to the interaction between PML-RAR α and the endogenous EZH2/EED/SUZ12 complex since there is almost no HMT activity in untransfected cells (lane 1). Moreover, lysates of 293T cells expressing Flag-PML-RAR α and the PRC2 complex were immunodepleted using mouse anti-EZH2 antibody or anti-SUZ12 antibodies. Under these conditions, we detected only a very low H3 methylation activity associated with PML-RAR α (Figures 2C and 2D and Figures S3B and S3C), likely due to residual PRC2/3/4 complexes or to the association with other SET domain containing proteins (Carbone et al., 2006).

We further tested whether this activity requires the SET domain of EZH2 by expressing the Δ SET-EZH2 protein together with the other components of the PRC2 complex (Figure 2B and Figure S3A, lane 4). The subsequent PML-RAR α /PRC2 immunopurified complex contained the Δ SET-EZH2 protein but had a strongly reduced HMT activity on histone H3 when compared to that containing wild-type EZH2 (lanes 3 and 4). Thus, these studies reveal that the oncogenic transcription factor PML-RAR α interacts with and may recruit the PRC2/3/4 complexes to its target promoters.

Next we wanted to explore the possibility that PML-RAR α could recruit the PRC2/3/4 complexes to one of its target promoters. Chromatin immunoprecipitation (ChIP) experiments for SUZ12 were performed in U937-PR9 cells in the absence or presence of PML-RAR α (i.e.

to activate endogenous RARs (Di Croce et al., 2002), and then with 100 μ M Zn to induce PML-RAR α expression, were subjected to ChIP analysis using the indicated antibodies. The promoter of RAR β 2 was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments.

prior to or after Zn²⁺ induction). SUZ12 was found to be associated with RAR β 2 promoter only in cells expressing PML-RAR α (Figure 2E). Consistent with the binding of PRC2/3/4 to the RAR β 2 promoter, H3K27me3 was significantly increased in those cells (Figure 2E). No enrichment was detected using an unrelated antibody. Together, these data strongly suggest that the PRC2/3/4 complex is found in complexes with PML-RAR α at RAR β 2 promoter.

SUZ12 Knockdown Affects PcG-Dependent Epigenetic Marks in APL

We and others have recently demonstrated that loss of SUZ12 expression both *in vivo* and in tissue culture cells strongly impaired the activity of EZH2 (Cao and Zhang, 2004a; Pasini et al., 2004), suggesting that SUZ12 may be required for the stability of the PRC2/3/4 complexes. Thus, in order to analyze the role of the PRC2/3/4 complexes in PML-RAR α -dependent gene silencing, we generated a stable SUZ12 knockdown NB4 cell line (RNAi SUZ12) using a retroviral vector-based shRNA approach (Brummelkamp et al., 2002). A reduction of >75% of SUZ12 protein was achieved under these conditions when compared to the mock knockdown cells (RNAi control) (Figure 3A, left panel), and a corresponding decrease in bulk histone H3 tri-methyl K27 level (H3K27me3) was observed (Figure 3A, left panel) in agreement with previous data (Cao and Zhang, 2004a), while other lysines modifications were not affected (H3K9me3, H4K20me3, H3K4me3) (data not shown).

We next tested by ChIP coupled with quantitative PCR how SUZ12 knockdown affects H3K27 modifications and promoter activity of the RAR β 2 promoter. H3K27me2 and H3K27me3 marks are significantly reduced in RNAi SUZ12 cells when compared to mock knockdown cells concomitantly with an increase of H3K27me1 and H3K27ac marks (Figure 3B). Importantly, EZH2 was displaced from RAR β 2 promoter in RNAi SUZ12 cells. These results are in line with the proposed role of the PRC2 complex in catalyzing the conversion of monomethylated K27 to the di-/tri-methylated forms (Cao and Zhang, 2004b; Shilatifard, 2006). As controls for the ChIP assays and for antibody specificity, equal amounts of unrelated antibody (HA) were also included. As an additional control, we performed ChIP assays for SUZ12 (Figure 3A, right panel). A reduction in the occupancy of SUZ12 on the RAR β 2 promoter was observed in knockdown cells when compared to mock knockdown cells or to the parental NB4 cells. Thus, in PML-RAR α -expressing cells, the PRC2/3/4 repressor complexes are recruited to the RAR β 2 promoter and dictate the epigenetic state of K27 modification.

To strengthen our observations, we expanded our analysis to other PML-RAR α target genes that could be potentially coregulated by the PRC2/3/4 complexes. Recently, global gene expression studies identified several PML-RAR α regulated genes (Meani et al., 2005). Among those that are significantly downregulated following the expression of the fusion protein (Carbone et al., 2006), we characterized the hPSCD4 and hNFE2 promoters

based on the presence of several RARE elements within the regulative promoter regions. ChIP experiments confirmed that similar to RAR β 2, hPSCD4 and hNFE2 promoters showed a reduction of H3K27me2 and H3K27me3 epigenetic marks concomitantly with increase acetylation of H3K27+ in cells lacking functional PRC2 complex (Figures S4A and S4B).

Although the PcGs and DNMTs originally were believed to function in independent epigenetic memory systems, we have recently documented the presence of a complex containing both activities (Vire et al., 2006). To extend this observation to the silencing of cellular promoters by oncoproteins, we tested whether the DNA methylation levels of the RAR β 2 promoter were altered in cells lacking the PRC2/3/4 complexes. Thus, we performed bisulphite genomic sequencing analysis in the same experimental setting described above. As previously reported, the RAR β 2 promoter presented high levels of DNA methylation in wild-type or mock-infected leukemic cells (Figures 3C and 5A) (Di Croce et al., 2002). Interestingly, in SUZ12 knockdown cells, methylation of CpGs was reduced by 50% (Figure 3C). In agreement with these results, we found that the occupancy of DNMT1 and DNMT3b to the RAR β 2 promoter was lower in the absence of PRC2/3/4 (Figure 3B), and that this was not caused by a diminished amount of DNMTs in the cells (data not shown).

Since the two main epigenetic-repressive marks are reduced in the NB4 knockdown cells, we next analyzed the levels of RAR β 2 messenger RNA in these cells. By performing RT-qPCR, we detected elevated transcript levels of RAR β 2 in cells lacking a functional PRC2/3/4 complexes (Figure 3D).

Parallel experiments performed on the HL60 cell line (with no PML-RAR α) strongly suggested that the above results are specific for the presence of PML-RAR α . Specifically, knockdown of SUZ12 expression in HL60 cells (Figure S5A) does not alter any of the epigenetic marks that were observed to be affected at PML-RAR α target genes in NB4 cells, namely H3K27me2, H3K27me3, and H3K27ac (Figure S5B). Similarly, knockdown of SUZ12 in HL60 did not affect the binding of DNMT3a/b at RAR β 2 promoter (Figure S5B). We then analyzed by bisulphite genomic sequencing the methylation status of the RAR β 2 promoter in HL60 cells with or without SUZ12. The level of methylated CpGs in control HL60 cells is similar to that observed in NB4 cells; however, knockdown of SUZ12 does not cause any decrease of DNA methylation in HL60 cells (Figure S5C) in contrast to the 50% reduction observed in NB4 SUZ12 RNAi. Finally, we analyzed by RT-PCR the transcriptional activity of the RAR β 2 gene in HL60 RNAi control compared to RNAi SUZ12, and we did not observe any promoter derepression (data not shown).

SUZ12 Knockdown Facilitates Cellular Differentiation

We next investigated whether the epigenetic and transcriptional changes at the promoter level due to the SUZ12 knockdown could in turn affect global cell differentiation. We quantified the levels of CD11b and CD11c

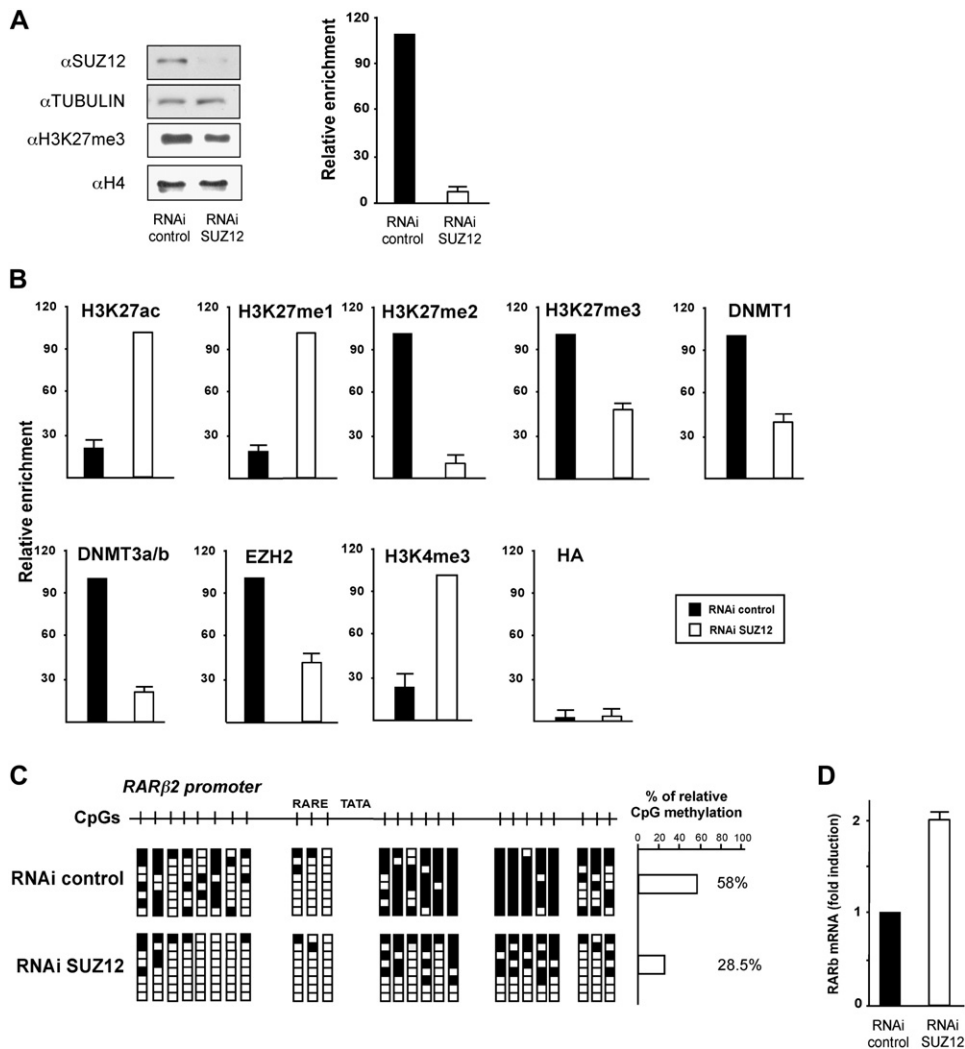


Figure 3. Effects of PcG Depletion in NB4 Leukemic Cells

(A) Knockdown of SUZ12 impairs the PcG complex activity. Human NB4 leukemic cells were infected with a retroviral construct generating SUZ12-specific small hairpin RNA (RNAi SUZ12) or the scrambled control sequence (RNAi control) and selected with puromycin for 3 days. Equal amounts of cell extracts from mock and interference RNA cells were blotted with the indicated antibodies (left panel). Specific binding of SUZ12 on the RARβ2 promoter in NB4 cells; RNAi SUZ12 or control cells were subjected to ChIP analysis using an antibody against SUZ12. The promoter of RARβ2 was amplified with real-time PCR (right panel). Error bars indicate the standard deviation obtained from three independent experiments.

(B) Downregulation of SUZ12 causes changes in the pattern of histone modifications, EZH2, and DNMTs occupancy on PML-RARα target genes. RNAi SUZ12 or control cells were subjected to ChIP analysis as in Figure 3A, using the indicated antibodies.

(C) Impairing PcG activity reduces DNA methylation of the RARβ2 promoter. The RARβ2 5' region in RNAi SUZ12 or RNAi control cells was subjected to bisulfite genomic sequencing. The methylation status of each CpG dinucleotide in each sequenced cloned is depicted by a black square if the position was methylated or a white square if it was not.

(D) Impairing PcG activity reactivates RARβ2. Total RNA was prepared from RNAi SUZ12 or RNAi control cells after treatment with suboptimal amount of RA (1 nM), and RARβ2 gene expression was analyzed relative to actin control by quantitative real-time PCR. Results are expressed as the mean ±SEM of two independent experiments performed in duplicate.

differentiation marker by flow cytometry as an indicator of maturation of the hematopoietic cells (Lanotte et al., 1991). CD11c level was increased by more than 2-fold in the absence of functional PRC2/3/4 complexes (data not shown). Interestingly, the simultaneous addition of a differentiation stimulus (in this case, retinoic acid [RA], see below) synergized in inducing cell differentiation (Figures S4C and S4D). Cell differentiation was also evaluated

in all samples after Wright-Giemsa staining (Figure 4A). Relative to NB4 RNAi control cell, SUZ12 RNAi displayed a more mature morphology as observed by several features: (1) they contained a reduced nucleus-to-cytoplasm ratios; (2) the dark blue-gray cytoplasm became lighter and often contained granules; and (3) they frequently displayed indented-shaped nuclei and an aggregated pattern of nuclear chromatin with an irregular cellular shape.

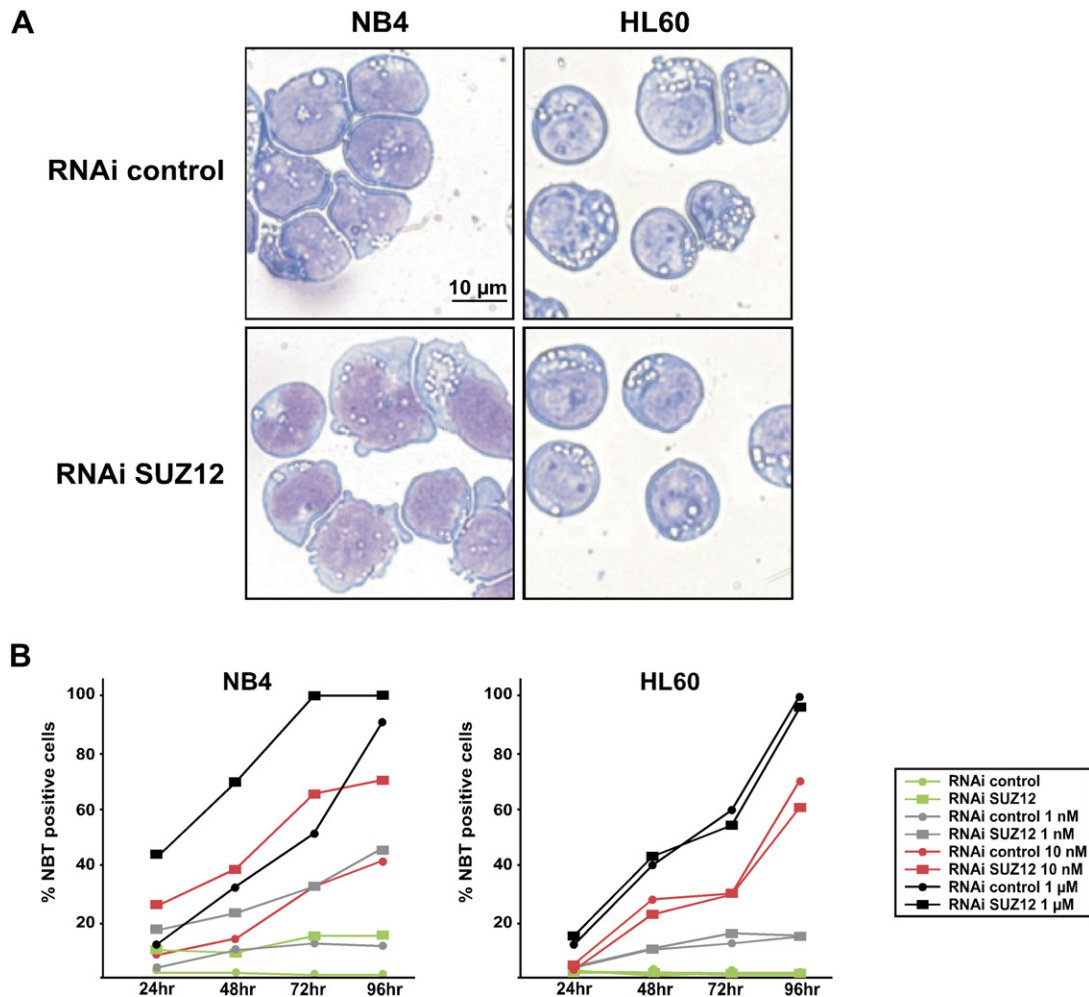


Figure 4. Impairing of PcG Activity Facilitates the Differentiation of NB4 Leukemic Cells

(A) Morphologic differentiation of SUZ12 knockdown NB4 and HL60 cells. Leukemic cells were stained with Wright-Giemsa and analyzed for the morphology under the light microscopy.

(B) Kinetics of differentiation of SUZ12 knockdown NB4 and HL60 after treatment with different concentration of RA. Leukemic cells were assayed for NBT reduction and counted under the light microscope. Standard deviation was <5%.

These differences were much more pronounced in untreated cells, while RA administration, as expected, induced differentiation in both samples.

We also monitored NB4 maturation by measuring the capacity of differentiated cells to reduce nitroblue tetrazolium (NBT). Examination of the same samples as used for the morphology (see above) revealed little evidence of spontaneous differentiation in untreated control RNAi cells, while we observed a significantly increased number of NBT-positive cells in RNAi SUZ12 NB4 (Figure 4B). Extensive kinetic studies of RA administration confirmed that in cells lacking SUZ12, a more pronounced differentiation (up to 3-fold) was observed (Figure 4B), likely reflecting the differences in untreated cells.

We extended our analysis to control and SUZ12 RNAi HL60 cells in order to properly compare the observed effects in a leukemic cell line that does not express PML-RAR α . No differences between HL60 control RNAi

and SUZ12 RNAi cells were observed for differentiation-associated antigens, morphology, or NBT reduction (Figures 4A and 4B and Figure S4E).

In summary, these results suggest that PML-RAR α represses its target genes in a PRC2/3/4-dependent manner. The presence of both PRC2/3/4 as well as DNMTs is required for efficient gene silencing. Moreover, SUZ12 knockdown NB4 cells are sensitized, and thus more prone to differentiation.

PcG-Dependent Epigenetic Marks and DNA Methylation

To investigate a potential crosstalk between DNA and H3K27 methylation at PML-RAR α -silenced promoters, we tested whether inhibition of DNA methylation would change the methylation status of histone H3 associated with the promoter. We incubated NB4 cells for 48 hr with 5-aza-2'-deoxycytidine (5-aza-dC), which causes

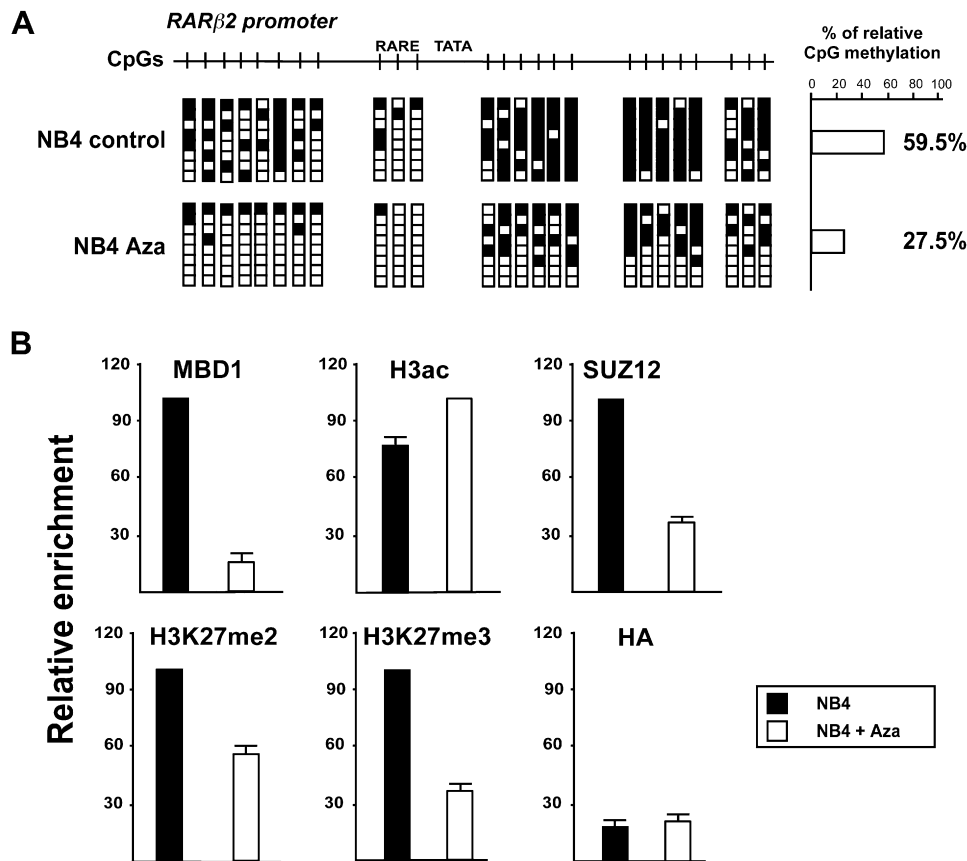


Figure 5. Crosstalk between DNA Methylation and Histone Methylation in NB4 Leukemic Cells

(A) Bisulfite genomic sequencing of the *RARβ2* 5' region in NB4 cells. The methylation status of each CpG dinucleotide in each sequenced clone is depicted by a black square if the position was methylated or a white square, if it was not. Where indicated, cells were treated with 0.1 μM 5-Aza-dC for 48 hr.

(B) Loss of DNA methylation destabilizes the PcG complex. 5-Aza-dC or mock-treated NB4 cells were subjected to ChIP analysis using the indicated antibodies. The promoter of *RARβ2* was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments.

irreversible inhibition and degradation of DNMTs but does not affect PML-RAR α and EZH2 protein levels. 5-aza-dC treatment induced *RARβ2* promoter demethylation, as compared to mock-treated cells (Figure 5A). We have previously demonstrated that the methyl-binding domain containing protein 1 (MBD1) is specifically associated with *RARβ2* hypermethylated promoter (Villa et al., 2006). In 5-aza-dC treated cells, we found a 5-fold reduction of MBD1 bound to the PML-RAR α target gene (Figure 5B) in agreement with promoter hypo-methylation (Figure 5A). We next asked whether 5-aza-dC treatment could also affect binding of PRC2/3/4 complexes. Indeed, both SUZ12 binding and presence of di- and trimethylated H3K27 were reduced with the DNMT inhibitor (Figure 5B), concomitantly with a reduction in H4K20me3, while other repressive marks were relatively unaffected (H3K9me3 and H4K20me2, Figure S6A). Importantly, we did not observe any significant variation in HL60 cells treated or not with 5-aza-dC (Figure S6C). Of note, the ChIP data for SUZ12 suggested that very small fraction, if any, was bound to *RARβ2* promoter. Similarly,

H3K27me3 signal at *RARβ2* promoter was much lower if compared to NB4 cells. These data are consistent with the incapability of endogenous wild-type RAR to recruit PRC2/3/4 complexes to its target genes and with the binding of SUZ12 in cell specific manner (Squazzo et al., 2006). These results, together with those described above, unveil a crosstalk between DNA methylation and H3K27 methylation in PML-RAR α -expressing leukemic cells. Perturbation of one of the two key epigenetic cellular memory systems affects the other as well, thus supporting the hypothesis that a continuous feedback loop is required for aberrant gene silencing of tumor suppressors in cancer cells.

Epigenetic Silencing Marks Are Erased by RA Treatment

Human APL patients are treated during the early phase of the disease with pharmacological doses (10⁻⁶ M) of RA (Di Croce, 2005; Licht, 2006), which are needed to dissociate the NCoR/HDAC3 complex from PML-RAR α and to recruit histone acetyltransferase (HAT) enzymes. We, as well

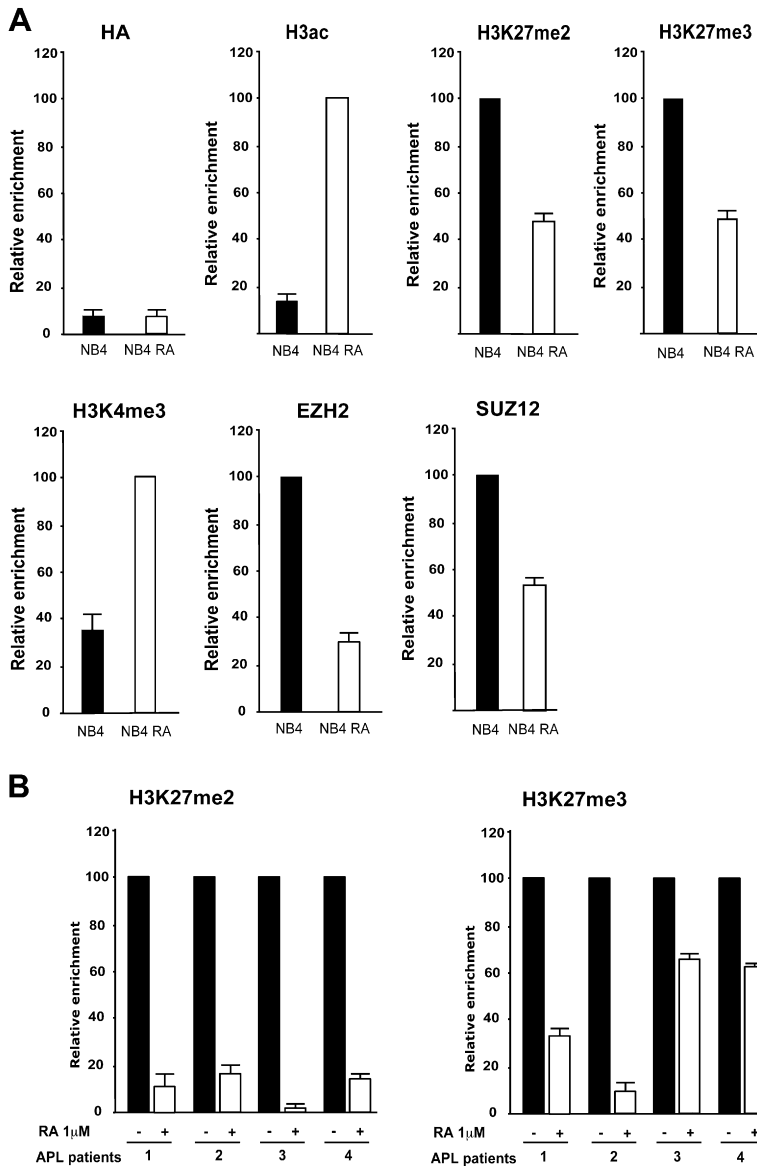


Figure 6. PML-RAR α Target Genes Are Methylated at H3K27 in APL Cells

(A) RAR β 2 promoter is methylated at H3K27 in NB4 cells. RA (1 μ M) or mock-treated NB4 cells were subjected to ChIP analysis using the indicated antibodies. The promoter of RAR β 2 was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments.

(B) The RAR β 2 promoter is methylated at H3K27 in primary blasts from patients. APL primary blasts treated with RA (1 μ M, 48 hr) or mock-treated were subjected to ChIP analysis using the indicated antibodies. The promoter of RAR β 2 was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments.

others, have previously demonstrated that under similar conditions, RA reduces promoter methylation at PML-RAR α target genes, including RAR β 2, leading to promoter reactivation in both NB4 and primary blast isolated from APL patients (Di Croce et al., 2002; Fazi et al., 2005). In order to further characterize the effect of RA on the epigenetic silencing imposed by PML-RAR α , we performed ChIP assays in NB4 cells treated or not with RA. RA administration caused a reduction of SUZ12 and EZH2 bound to RAR β 2 promoter (Figure 6A), with a corresponding decrease of H3K27me2 and H3K27me3 epigenetic marks at the same promoter region. Interestingly, the acetylation levels of histone H3 and H3K4me3 were significantly higher after RA treatment (Figure 6A), consistent with promoter reactivation, while levels of H4K20me2 and H4K20me3 were unaffected (Figure S7B). ChIP results obtained in untreated or RA-treated HL60 cells

revealed only very small variations in all the epigenetic marks (H3ac, H3K27me2, H3K4me3) and PcG proteins (SUZ12) analyzed (Figure S7C). Thus, taken together with our previous results, this indicates that pharmacological doses of RA reverts the epigenetic silencing and the promoter dormancy imposed by PML-RAR α in human-derived NB4 cell line through the erasure of specific epigenetic marks.

Next we wanted to investigate whether primary blasts isolated from APL patients presented a similar epigenetic profile for H3K27 to those observed in NB4 cells, and whether pharmacological doses of RA could have any effect. ChIP experiments were performed on isolated cells from four newly diagnosed APL patients treated or not with RA for 48 hr. As shown in Figure 6B, untreated leukemic cells showed high levels of methylated H3K27 over the RAR β 2 promoter regions, while RA treatment

significantly reduced these levels. The capability of RA to reduce H3K27me3 was also confirmed for other PML-RAR α target genes (Figure S7A). Our results obtained using leukemic cell lines and ex vivo blasts from APL patients suggest that RA administration at concentrations used for patient therapy reverted most of the epigenetic silencing imposed by PML-RAR α .

DISCUSSION

In *Drosophila*, PcG mediates silencing through binding to cis-acting PcG-responsive elements (PREs) (Lund and van Lohuizen, 2004b). Although in mammals several PcG target genes have been identified, no mammalian PRE has been described to date. Thus, PcG targeting may relay on direct (Garcia et al., 1999; Satijn et al., 2001) or indirect (Epping et al., 2005) interactions with transcription factors to dictate target gene specificity. Our data unveil a mechanism for carcinogenesis. We demonstrated that PcG-mediated histone modifications contribute to tumor suppressor gene silencing. Importantly, the oncogenic protein PML-RAR α was found associated with members of the PRC2/3/4 complexes. In leukemic cells, PML-RAR α -mediated targeting of PRC2/3/4 to tumor suppressor genes contributes to promoter silencing and cancer progression. Indeed, upon PML-RAR α expression, SUZ12 and methylated H3K27 were found at the RAR β 2 promoter. Importantly, knockdown of SUZ12 in leukemic cells led to a reduction of the H3K27 epigenetic marks at PML-RAR α target genes, which reverberated on the DNA methylation levels.

PML-RAR α oligomerization allows binding to a variety of DNA response elements that are not regulated by RAR α (Kamashev et al., 2004) and is an essential event for the leukemogenesis process. Interestingly, recent data suggested that forced oligomerization of RAR α is insufficient for triggering APL in vivo, thus unveiling an important role of the PML moiety in APL (Kwok et al., 2006; Sternsdorf et al., 2006). The PML protein has been implicated in a large number of biological processes, including cell-cycle control, myeloid differentiation, apoptosis, and has tumor growth suppressor properties. Elegant experiments have demonstrated that mutation of K160 of PML, which correspond to the major site of sumoylation, abrogates the ability of PML-RAR α to initiate leukemia in vivo by affecting the recruitment of Daxx repressor (Zhu et al., 2005). Similarly, our data suggest that between the two moieties, PML revealed stronger affinity for PRC2/3/4 complexes, likely allowing and/or stabilizing the interaction between PML-RAR α and Polycomb complexes. Given the link between Daxx and DMAP1/DNMTs (Muramoto et al., 2004) and between PRC2 and DNMTs (Vire et al., 2006), it would be interesting to investigate whether the K160 mutation also affects PRC2 recruitment and epigenetic marks at PML-RAR α target genes.

Links between histone modifications and DNA methylation were initially described few years ago when several groups reported the association of DNMTs with HDAC

enzymes (Fuks et al., 2000; Robertson et al., 2000) and with SUV39H1 (Fuks et al., 2003). More recently, Vogelstein and coworkers have reported that H3K9 methylation can occur independently of DNA methylation at p16^{INK4a} gene in HCT116 cells (Bachman et al., 2003). Here, we have shown that both Polycomb-dependent H3K27 and DNA methylation are required for the maintenance of the aberrant epigenetic silencing and identify PML-RAR α as a paradigm for oncoproteins that are able to direct several epigenetic modifier enzymes to tumor suppressor and differentiation-related genes. These findings are of particular interest since both of these epigenetic memory systems have been linked to cancer, although independently. Indeed, DNA hypermethylation of cancer-related genes has been largely documented (Baylin and Bestor, 2002; Plass, 2002). On the other hand, PcG complexes were originally identified as epigenetic gene silencers during embryogenesis. Recent data have demonstrated that PcG proteins, e.g., BMI1 or EZH2, are overexpressed in human tumors (reviewed in Lund and van Lohuizen, 2004a), but to date, mechanistic insight into the function of PRC2/3/4 in cancer cells is lacking. Thus, our data represent a major advance toward understanding how leukemogenesis can be induced by a modified transcription factor (the PML-RAR fusion oncoprotein) via its ability to recruit Polycomb proteins to gene promoters. Given the potential crosstalk among several epigenetic marks, we are currently investigating the precise kinetics among histone modifications (such as H3K9 [Carbone et al., 2006] and H3K27 [this manuscript]) and DNA methylation (Di Croce et al., 2002) and their relative contribution to the silencing mechanism during early steps of leukemogenesis.

The presence of H3K27 methylation not only reinforces the silencing imposed by DNMTs but may also facilitate binding of other repressor Polycomb complex, e.g., PRC1 (Kuzmichev et al., 2004). Indeed, our preliminary data suggest that PRC1/PRC3 might allow further histone modifications, as well as deposition and posttranslational modification of histone H1 (data not shown). A chain of epigenetic events (that include epigenetic-silencing marks, binding of further repressor proteins, and histone H1 and chromatin condensation) initiated by the binding of PML-RAR α to its target genes might ensure irreversible epigenetic silencing, which cannot be overcome in a physiological environment. Interestingly, in APL patient-derived cell lines as well as in freshly isolated blasts, supraphysiological concentrations of retinoic acid, which are used as an initial therapy for APL patients, can restore the proper balance of epigenetic marks, favoring the cellular differentiation toward mature neutrophil granulocytes. A major impairment to successfully treating APL patients is RA resistance, which often arises due to acquired resistance to the hormone, with consequent relapse of the disease. Therefore, identification of agents that can effectively synergize with or provide an alternative to RA, and to chemotherapeutic agents in general, is of paramount relevance. Since epigenetic changes are potentially reversible, they make attractive targets for therapeutic intervention. Thus, our finding that inactivating

a key component of the Polycomb complex causes the reversal of these modifications and of the leukemic phenotype suggests the possibility that specific HMT inhibitors for EZH2 can be used in cancer therapy, opening a new avenue for therapeutic intervention.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

All plasmids encoding proteins of the PRC2 complex have been previously described (Pasini et al., 2004). The Suz12-specific sequence (AAGCTGTTACCAAGCTCCGTG, see also Pasini et al., 2004) was used to synthesize oligonucleotides to clone into pRetroSuper (Brummelkamp et al., 2002). The expression vectors for PML-RAR α , PML, RAR α , and p53-RAR α were previously described in Di Croce et al., 2002.

We used the following antibodies in this study: PGM3 (Di Croce et al., 2002) PML-RAR α , anti-RAR α (Santa-Cruz, sc-551), anti-HA (Roche), anti-SUZ12 (Abcam, Upstate), anti-Flag (Sigma-Aldrich), mouse anti-EZH2 BD43 (Pasini et al., 2004), mouse anti-EZH2 AC22 (Pasini et al., 2004) and mouse anti-EED AA19 (Bracken et al., 2003). To detect tubulin and H4 we used anti-tubulin (Abcam) and anti-H4 (Upstate), respectively. ChIP experiments were performed using anti-SUZ12 (Upstate), anti-Ezh2 AC22, anti-H3K27ac (Upstate), anti-H3K27me1 (Upstate), anti-H3K27me2 (Upstate), anti-H3K27me3 (Upstate), anti-H3K4me3 (Abcam), anti-H3K9me3, anti-H4K20me3, anti-H4K20me2 (Schotta et al., 2004), anti-DNMT1 (Imgenex), anti-DNMT3a/b (Santa Cruz), anti-MBD1 (Imgenex), and anti-H3ac (Upstate). For FACS analysis we used antibodies that recognize cell surface myeloid-specific antigens CD11c (Dakocytometri) and CD11b (Becton Dickinson).

Cell Lines, Transfection, and Retroviral Infection

NB4 cells, U937-PR9, HL60, and APL blasts from patient were cultured at 37°C and 5% of CO₂ in RPMI medium supplemented with 10% of fetal bovine serum. HEK 293T were cultured at 37°C and 5% of CO₂ in DMEM medium supplemented with 10% fetal bovine serum and then transfected using standard techniques. Cell extracts were prepared as described in Di Croce et al., 2002.

HEK 293T were transfected using the calcium phosphate coprecipitation method (Villa et al., 2006). pRS-based retrovirus was produced by transfecting GP2-293 packaging cells (Clontech). The collected retrovirus was subsequently used to transduce NB4 or HL60 cells by spinoculation (900 g, 90 min, 32°C) in presence of protamine sulfate followed by an additional overnight incubation at 37°C in 5% CO₂. The protocol was repeated for 2 consecutive days.

Clinical Samples

Studies involving human subjects were done in accordance with the IMAS Ethical Committee (Barcelona, Spain). Leukemic blasts from bone marrow and/or peripheral blood were obtained after informed consent from 15 newly diagnosed APL patients showing 75% leukemic infiltration. Blasts isolation and molecular characterization of the APL-associated PML-RAR isoforms (bcr1, bcr2, and bcr3) were performed as described (Cheson et al., 2003). Immunophenotyping study was performed at diagnosis following the methods reported in (Cheson et al., 2003). The fraction of malignant cells was always $\geq 70\%$ as determined by flow cytometry. DNA and RNA obtained from leukemic samples at diagnosis were assayed for PML-RAR α . Leukemic cells were cryopreserved after Ficoll-Paque gradient (Amersham, Uppsala, Sweden).

Protein Purification and PRC2 Immunodepletion

For in vitro binding assay, Flag-PML-RAR was immunopurified from 293T cells using anti-Flag agarose beads. Lysis of 293T cells and immunopurification was performed in high salt extraction buffer (50 mM Tris-HCl pH7.5, 300 mM NaCl, 5% Glycerol, 0.5% IGEPAL [Sigma],

1 mM EDTA) in order to minimize the interaction of PML-RAR with endogenous proteins. The PRC2 complex was expressed and purified as previously described (Pasini et al., 2004).

293T cells expressing Flag-PML-RAR α and the PRC2 complex were lysed in lysis buffer (Villa et al., 2006). Equal amount of protein lysate (1 mg) was incubated with protein-G beads (Amersham Biosciences) precoupled to mouse IgG as control and to mouse anti-EZH2 or anti-SUZ12 antibody. Not bound fractions were collected and filtered to avoid carry over of protein G beads. Immunodepleted lysates were used for PML-RAR α associated HMT activity assays.

Histone Methyltransferase Assay

Protein lysates were incubated for 4 hr with Anti-Flag Agarose beads (Sigma-Aldrich). Beads were extensively washed with lysis buffer (Villa et al., 2006) and assayed for HMT activity in the presence of oligonucleosomes as substrate. HMT assays and Hela oligonucleosomes preparation were performed as previously described (Pasini et al., 2004).

Immunoprecipitation and ChIP

For coimmunoprecipitation assay, cells were washed in PBS and lysed in lysis buffer (Villa et al., 2006). Specific antibodies were added to the lysates from 3 hr up to 16 hr with 20 μ l of slurry protein A Sepharose beads (Amersham Biosciences) saturated with BSA. The immunoprecipitates were then washed and loaded on SDS/PAGE.

For chromatin immunoprecipitation (ChIP), NB4, U937-PR9, HL60, or primary cells from patients were crosslinked with 0.8% of formaldehyde at room temperature for 6 min, and the reaction was stopped by the addition of glycine (0.125 M). Cells were rinsed two times with cold PBS, resuspended in lysis buffer (1% SDS, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8, P.I.) and sonicated with the Bioruptor (Biogenide). Lysates were diluted ten times with the IP buffer (1% TRITON X-100, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, P.I.) and then incubated overnight with 5 μ g of each antibody. 40 μ l of protein A sepharose beads saturated with salmon sperm (Upstate) were added to the lysates for 2 hr and then washed for four times with Wash buffer 1 (1% TRITON X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, P.I.) and one time with Wash buffer 2 (1% TRITON X-100, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, P.I.). The immunoprecipitated DNA was quantified by real-time quantitative PCR (Roche LightCycler). Quantification of ChIP results was performed relative to the input amount according to Frank et al., 2001 and setting the reference samples to the arbitrary value of 100. The sequences of the PCR primers are available upon request.

Bisulphite Genomic Sequencing

Bisulphite genomic sequencing was performed on bisulphate-treated genomic DNA as described in Di Croce et al., 2002.

RNA Preparation and Analysis

RNA, from NB4 Suz12 interference RNA cells (RNAi Suz12) or from NB4 control cells (RNAi control) after RA treatment (1 nM, 6 hr), was extracted (QIAGEN Rneasy mini kit), retrotranscribed (AMV, Roche), and assayed for the expression of RAR β 2 using quantitative real-time PCR (Roche LightCycler). The sequences of the PCR primers are available upon request.

Cell Differentiation

NB4 and HL60, after treatment with 1 μ M of RA where indicated, were rinsed twice with PBS and incubated 30 min with specific antibodies. After washing three times with PBS, the percentage of differentiated antigen-positive cells and the fluorescence were analyzed by flow cytometry on Becton Dickinson FACSCanto.

The nitroblue tetrazolium (NBT) assay was performed using a commercially available NBT (Sigma). Two-hundred microliters of cell suspension at a density of 2×10^5 cells were mixed with 200 μ l of filtered 0.2% NBT solution and 3 μ l of TPA (1 μ M) and further incubated for 30 min at 37°C. Subsequently, cytocentrifuge slides were prepared

(200 rpm, 4 min). NBT positive cells were determined by scoring 400 cells under a light microscope. Where indicated, slides were also stained for 1 min with modified Wright-Giemsa stain (Sigma), rinsed in PBS, stained 10 min in modified Giemsa stain (Sigma) diluted 1:20 with water (Sigma), rinsed with water, and subjected to morphological examination under a light microscopy.

Supplemental Data

The Supplemental Data include seven supplemental figures and can be found with this article online at <http://www.cancer-cell.org/cgi/content/full/11/6/513/DC1/>.

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