The methyl-CpG binding protein MBD1 is required for PML-RARα function

Raffaella Villa*, Lluis Morey*, Veronica A. Raker*, Marcus Buschbeck*, Arantxa Gutierrez*, Francesca De Santis†, Massimo Corsaro†, Florencio Varas*, Daniela Bossi†, Saverio Minucci†, Pier Giuseppe Pelicci†, and Luciano Di Croce*‡§

*Centre de Regulacio Genomica, Universitat Pompeu Fabra, Passeig Maritim 37-49, 08003 Barcelona, Spain; †European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy; and ‡Institució Catalana de Recerca i Estudis Avançats and Centre de Regulacio Genomica, Passeig Maritim 37-49, 08003 Barcelona, Spain

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PML-RARα induces a block of hematopoietic differentiation and acute promyelocytic leukemia. This block is based on its capacity to inactivate target genes by recruiting histone deacetylase (HDAC) and DNA methyltransferase activities. Here we report that MBD1, a member of a conserved family of proteins able to bind methylated DNA, cooperates with PML-RARα in transcriptional repression and cellular transformation. PML-RARα recruits MBD1 to its target promoter through an HDAC3-mediated mechanism. Binding of HDAC3 and MBD1 is not confined to the promoter region but instead is spread over the locus. Knock-down of HDAC3 expression by RNA interference in acute promyelocytic leukemia cells alleviates PML-RAR-induced promoter silencing. We further demonstrate that retroviral expression of dominant-negative mutants of MBD1 in hematopoietic precursors compromises the ability of PML-RARα to block their differentiation and thus restored cell differentiation. Our results demonstrate that PML-RARα functions by recruiting an HDAC3–MBD1 complex that contributes to the establishment and maintenance of the silenced chromatin state.

chromatin | epigenetics | leukemia

In mammalian cells, DNA methylation occurs predominantly at CpG dinucleotides, which are distributed unevenly and are underrepresented in the genome. Clusters of usually unmethylated CpGs (termed CpG islands) are found in many promoter regions (reviewed in ref. 1). Changes in DNA methylation leading to aberrant gene silencing have been demonstrated in several human cancers (2). Hypermethylation of promoters was demonstrated to be a frequent mechanism leading to the inactivation of tumor suppressor genes (3).

DNA methylation leads to gene silencing by means of two distinct mechanisms: (*i*) methylation at CpG sites that prevents binding of transcription factors and (*ii*) recognition of mCpGs by a family of methyl-CpG binding proteins (MBD). Among these proteins, MBD1 affects chromatin structure and gene silencing through a yet-unknown mechanism that likely involves histone deacetylases (HDACs) (4).

Acute promyelocytic leukemia (APL) is characterized by the 15;17 chromosome translocation (5). The t(15,17), which involves promyelocytic leukemia (PML) on chromosome 15 and the retinoic acid (RA) α receptor (RARα) on chromosome 17, generates the chimeric PML-RARα gene. In the absence of RA, wild-type RARs bind to specific DNA sequences called RA responsive elements (RARE) and are able to repress transcription by recruiting corepressor complexes such as SMRT/NCoR/HDAC (6, 7). Physiological concentrations of RA trigger the dissociation of corepressor complexes and allow for the recruitment of several coactivators, including histone acetylases. Consequentially, RA treatment leads to transcriptional activation. Finely tuned expression of RA-responsive genes is necessary for the appropriate differentiation of myeloid cell lineages. In contrast to wild-type RARα, the transforming protein PML-RARα is rendered insensitive to physiological concentrations of RA that would usually trigger transcriptional activation. Because of its oligomerization state (8), PML-RARα forms stable complexes with corepressors and with DNA methyltransferases (DNMTs) to target promoters (9), functioning as a constitutive and potent transcriptional repressor of RARE-containing genes. It is thus commonly accepted that APL is caused by the repressive function of PML-RARα. Treatment of APL patients with higher pharmacological doses of RA forces the release of corepressor complexes from promoters targeted by PML-RARα, thus promoting partial transcriptional derepression (10, 11).

Here we show that MBD1 and PML-RARα are both required for complete silencing of PML-RARα target genes. PML-RARα indirectly recruits MBD1 to its target promoter through an HDAC3-mediated mechanism. Mutations in the MBD domain and transrepression domain (TRD) of MBD1 restore transcriptional activity and prevent the PML-RARα-induced hematopoietic differentiation block. Together these results identify MBD1 as a critical mediator of PML-RARα-induced gene silencing subsequent to promoter hypermethylation.

Results

MBD1 Cooperates with PML-RARα in Repressing Promoter Activity. The oncogenic protein PML-RARα induces promoter hypermethylation at CpG dinucleotides by direct recruitment of DNMT enzymes (9). Because methylated CpGs are potential docking sites for the binding of MBD proteins, we wanted to investigate the role of MBD proteins in the PML-RARα-mediated transcriptional silencing. In PML-RARα-expressing cells, such as the hematopoietic precursor U937-PR9 cells and NB4 cells, MBD1 is the most abundant of the various MBDs (data not shown). Based on these results, we explored the possibility that MBD1 contributes directly to PML-RARα gene repression. For this purpose, we used the reporter plasmid pRARβ2-luc, which contains the firefly luciferase gene driven by a 5-kbp fragment of human RARβ2 promoter (9). After transient transfection, the RARβ2 gene promoter was silenced only when PML-RARα was expressed at high concentrations (Fig. 1*A*, lanes 1–3). We next investigated the contribution, if any, of MBD1 in the regulation of the RARβ2 promoter. Although neither high nor low concentrations of MBD1 alone influenced RARβ2 transcription (Fig. 1*A*, lanes 4 and 5), coexpression of MBD1 with suboptimal amounts of PML-RARα dramatically repressed the RARβ2 promoter (Fig. 1*A*, lane 6). Strikingly, a promoter previously methylated *in vitro* by SssI DNA methylase was similarly repressed in the presence of

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Abbreviations: PML, promyelocytic leukemia; APL, acute PML; DNMT, DNA methyltransferase; HDAC, histone deacetylase; MBD1, methyl-CpG binding protein; RA, retinoic acid; RAR, RA receptor; RARE, RA responsive element; ChIP, chromatin immunoprecipitation; TRD, transrepression domain; TSA, trichostatin A.

§To whom correspondence should be addressed. E-mail: luciano.dicroce@crg.es.

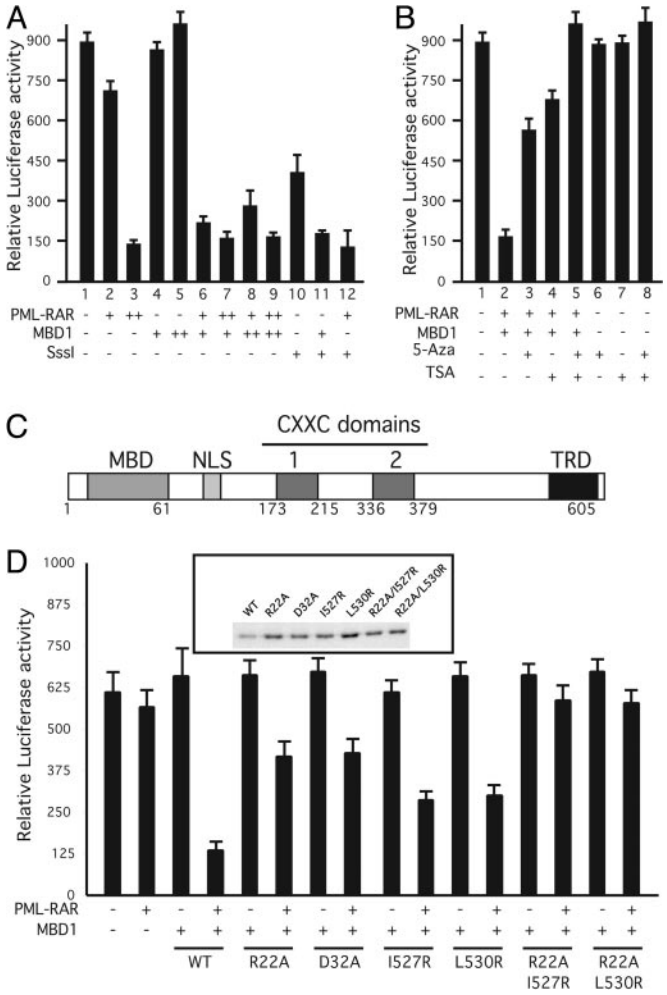

Fig. 1. MBD1 synergizes with PML-RARα to repress RARβ2 promoter activity. (*A*) Effect of MBD1 on PML-RARα-mediated repression of RARβ2. 293T cells were transfected with a reporter construct containing the human RARβ2 promoter upstream of the luciferase cDNA (0.5 μg) and expression vectors for PML-RARα (10 ng and 1 μg) and MBD1 (10 ng and 500 ng). Where indicated, the reporter construct was methylated *in vitro* with SssI before addition. Error bars represent the standard deviation from the mean for triplicate experiments. (*B*) Effect of DNA methylation and HDAC inhibitors on transcriptional repression by MBD1. TSA (100 nM) or 5-Aza-dC (1 μM), or a combination of these, was added 20 and 36 h, respectively, before the reporter assay was performed as described in *A*. Error bars represent the standard deviation from the mean for triplicate experiments. (*C*) Structure of MBD1. (*D*) Effect of the mutated MBD1 on PML-RARα-mediated repression. Luciferase assay was performed as in *A* by using expression plasmids for PML-RARα (10 ng), MBD1 (100 ng), and MBD1 mutants (100 ng). Error bars represent the standard deviation from the mean for triplicate experiments. (*Inset*) Wild-type and mutant forms of MBD1 are expressed at equal levels.

either MBD1 or PML-RARα (Fig. 1*A*, lanes 10–12). The synergistic repression mediated by MBD1 and PML-RARα was partially reversed by treating cells with trichostatin A (TSA) or 5-Aza-dC, inhibitors of HDACs and DNMTs, respectively (Fig. 1*B*, lanes 3 and 4). Notably, combination of both drugs completely restored RARβ2 promoter activity (Fig. 1*B*, lane 5), suggesting that PML-RARα-mediated repression involves histone deacetylation as well as CpG methylation, with subsequent binding of MBD1.

We have previously shown that PML-RARα causes RARβ2 promoter hypermethylation (9) (see also Fig. 5*B*, which is published as supporting information on the PNAS web site). We thus analyzed the contribution of MBD1 in PML-RAR-induced DNA methylation. Ectopic expression of PML-RARα recapitulated pRARβ2-luc hypermethylation (Fig. 5*A*). Interestingly, simultaneous expression of suboptimal concentration of PML-RARα (which alone has little to no effect on CpG methylation) together with MBD1 restored RARβ2 hypermethylation. However, combination of 5-Aza-dC and TSA almost completely prevented promoter methylation under similar conditions (Fig. 5*A*).

To understand whether PML-RARα binding to the RARβ2 promoter is a prerequisite for the synergistic repression with MBD1, we introduced three point mutations into the first half of the RARE, creating the ΔRARE reporter construct (Fig. 5*D*). It has been previously shown that these mutations impair the functionality of a murine RARE (12).

The down-mutation of RARE (ΔRARE) not only completely abolished PML-RARα-mediated repression of the RARβ2 promoter but also prevented its activation upon RA administration (Fig. 5*E*). Moreover, the ability of MBD1 to repress promoter activity synergistically with PML-RARα was strictly dependent on the presence of an intact RARE (Fig. 5*F*). Importantly, the integrity of the PML-RARα binding site was also a prerequisite for the induction of promoter methylation (Fig. 5*C*). Interestingly, MBD1 alone was able to repress the ΔRARE promoter when it had been previously methylated *in vitro*, suggesting that MBD1 can efficiently repress transcription once mCpGs are provided. Together, these results demonstrate that the repression of the RARβ2 promoter, mediated by PML-RARα and MBD1, is an active and specific mechanism, relying on binding of PML-RARα to RARE within the target promoter, with subsequent induction of promoter methylation and recruitment of MBD1.

Efficient MBD1 Corepression Activity Depends on the Integrity of both MBD1 and TRDs. We next wanted to study the contribution to gene silencing of the two well characterized domains of MBD1, the N-terminal methyl-CpG binding domain (MBD) and the C-terminal TRD (Fig. 1*C*). We generated MBD1 mutants in which either Arg-22 or Asp-32 was substituted with alanine. These particular amino acid substitutions were previously shown to abrogate the ability of the isolated MBD domain to bind to methylated DNA (13, 14). Each of the mutations in the MBD domain reduced the ability of MBD1 to corepress promoter activity with PML-RARα (Fig. 1*D*), indicating that binding of MBD1 to mCpGs is required to fully silence methylated promoters.

We next asked whether mutations in the TRD could also interfere with the ability of MBD1 to synergize with PML-RARα in gene repression. Bird and collaborators (15) identified the amino acids that are critical for the TRD to act as a transcriptional repressor. Based on these findings, we tested two mutant proteins, containing conversions of either isoleucine-527 or leucine-530 to arginine in their TRD, for their ability to repress promoter activity. Similar to the alterations in the MBD domain, these mutations reduced the repressive potential of MBD1 and allowed restoration of transcription from the RARβ2 promoter (Fig. 1*D*). A double-mutant MBD1 protein, defective in both MBD and TRDs (such as R22A and I527R or R22A and L530R) was completely unable to repress the RARβ2 promoter, suggesting that each of the two MBD1 domains analyzed in this study independently cooperates with PML-RARα in promoter silencing. All mutant proteins were expressed in the cell to a similar degree (Fig. 1*D Inset*).

HDAC3 Corepressor Complex Bridges PML-RARα and MBD1. It has been shown that both PML-RARα and MBDs proteins are associated with a TSA-sensitive deacetylase activity that is an important component of the repression mechanism (11, 15, 16). We thus predicted that an HDAC enzyme could interact with both proteins simultaneously. Because recent studies have sug-

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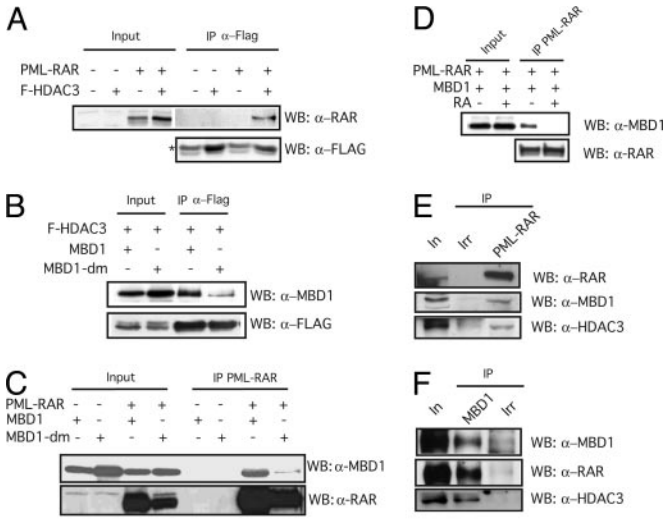

Fig. 2. HDAC3 bridges PML-RARα and MBD1. (*A*) Interaction between PML-RARα and HDAC3. 293T cells were transfected with PML-RARα and FLAG-HDAC3 expression vectors, and extracts were immunoprecipitated with anti-FLAG antibody. Western blots of input lysate or of immunoprecipitates were analyzed by using antisera against RARα or FLAG. Note that Flag-HADC3 comigrates with a nonspecific band (indicated by an asterisk) but is clearly seen above background levels. (*B*) Interaction between MBD1 and HDAC3. Cells were transfected with MBD1, MBD1-dm (MBD1 R22A/I527R), and FLAG-HDAC3 expression vectors, and extracts were immunoprecipitated with anti-FLAG antibody. Immunocomplexes were detected by Western blot as in *A*. (*C*) Interaction between MBD1 and PML-RARα. Cells were transfected with MBD1, MBD1-dm, and PML-RARα expression vectors, and extracts were immunoprecipitated with anti-PML (PGM3) antibody. (*D*) RA disrupts the interaction between PML-RARα and MBD1. 293T cells were transfected with MBD1 and PML-RARα expression vectors, and extracts underwent immunoprecipitation with an anti-PML (PGM3) antibody. Where indicated, cells were treated with RA (1 mM) 5 h before the immunoprecipitation was performed. (*E* and *F*) Endogenous interaction among PML-RARα, HDAC3, and MBD1. Cell extracts from NB4 were immunoprecipitated by using either PGM3 or anti-MBD1 antibody.

gested that the PML-RARα adapter proteins N-CoR and SMRT exist in a stable complex together with HDAC3 (17), we transiently transfected 293T cells with PML-RARα and Flag-tagged HDAC3 (F-HDAC3). After immunoprecipitation with anti-FLAG antibody, we found that HDAC3 was specifically associated with PML-RARα (Fig. 2*A*). Next, we analyzed the HDAC3–MBD1 interaction (Fig. 2*B*). Cells were cotransfected with F-HDAC3 and either wild-type MBD1 or MBD1 double mutant (MBD1-dm, R22A/I527R), as indicated. Immunoprecipitation results revealed that MBD1 associated with HDAC3 and that this association required the integrity of the TRD of MBD1. In contrast, mutations in the MBD domain (e.g., MBD1–R22A) did not affect MBD1–HDAC3 associations (data not shown). To determine the regions of HDAC3 that are crucial to the interaction with MBD1, GST-fused deletion mutants (or GST alone as a control) of HDAC3 were incubated with [^{35}S]methionine-labeled MBD1, produced by *in vitro* translation in a reticulocyte lysate. MBD1 was found to interact with the N-terminal region of GST-HDAC3 but neither with the C-terminal region nor with GST polypeptides alone (Fig. 6*A*, which is published as supporting information on the PNAS web site). To further confirm these results, several deletion mutants of FLAG-HDAC3 were expressed in 293T cells together with MBD1. Immunoprecipitation experiments revealed that the N-terminal region of HDAC3 mediates the interaction with MBD1 (data not shown).

Given the interaction between PML-RARα and HDAC3, and between HDAC3 and MBD1, we tested whether PML-RARα is associated with MBD1. In 293T cells, MBD1 could be coprecipitated with PML-RARα (Fig. 2*C*), and, likewise, PML-RARα could be coprecipitated with MBD1 (data not shown). The interaction resisted the presence of ethidium bromide in the precipitation reaction, thus excluding the possibility of a DNA-mediated protein association (data not shown). Little to no coprecipitation with PML-RARα was detected of a MBD1 protein mutated in the TRD, such as MBD1–I527R mutant (data not shown) or MBD1-dm (Fig. 2*C*). In contrast, a MBD1 protein bearing a mutation only in its MBD domain (R22A) was not altered in its ability to interact with PML-RARα (data not shown). Thus, PML-RARα can associate with MBD1, and this association requires the TRD.

We next wanted to identify which of the PML-RARα moieties mediates the interaction with MBD1. Association of MBD1 and RAR or ΔC-PML (which represents the PML part retained in the translocation) was analyzed by transient transfection experiments by using 293T cells. We additionally included in this study the chimeric protein p53-RAR (which contains the p53 tetramerization domain fused to RAR), because it has been shown to behave like PML-RARα in its capacity to block hematopoietic differentiation (8). Results from the corresponding coimmunoprecipitations revealed the existence of a stable complex of MBD1 with RAR (and p53-RAR) (Fig. 6*D*) but not with ΔC-PML (Fig. 6*E*), suggesting that recruitment of MBD1 by PML-RARα is mediated by its RAR moiety.

The PML-RARα–MBD1 association could be either direct or mediated through a common interacting partner, such as HDAC3. To analyze this association, we treated 293T cells with RA. The presence of this ligand at pharmacological doses is known to cause a conformational change in PML-RARα protein that leads to the release of the HDAC–corepressor complex, concomitantly with recruitment of coactivators (8). RA induced the release of MBD1 along with that of HDAC3 from PML-RARα (Fig. 2*D*), and the association between HDAC3 and MBD1 persisted even in the presence of RA (Fig. 5*G*). Thus, MBD1 association with PML-RARα depends on the simultaneous presence of HDAC complex, making it likely that HDAC3 bridges the two proteins.

To investigate whether PML-RARα associates with endogenous MBD1 and HDAC3, we performed coimmunoprecipitation experiments using lysates from either U937-PR9 cells or patient-derived NB4 cells. Immunoblot analysis of anti-PML-RARα immunoprecipitates revealed the existence of endogenous complexes of PML-RARα with MBD1 and with HDAC3 (Fig. 2*E*). Reverse experiments demonstrated that endogenous MBD1 associated with HDAC3 and PML-RARα (Fig. 2*F*). Together, these data strongly suggest that MBD1 and HDAC3 are found in complexes with PML-RARα.

HDAC3 Participates in PML-RARα-Dependent Repression of the Endogenous RARβ2 Promoter in APL Cells. Because HDAC3 appears to have an important structural role in the assembly of the PML-RARα repressor complex, we wondered whether HDAC3 could participate directly in gene silencing. We used interference RNA to reduce the expression of endogenous HDAC3 in NB4 leukemic cells. The sequence-specific short hairpin RNA vector pRS-HDAC3 reduced endogenous HDAC3 protein levels in human NB4 APL (Figs. 3*A* and 7*A*, which is published as supporting information on the PNAS web site). Under these conditions, endogenous RARβ2 mRNA was induced (Fig. 3*C*), concomitantly with an increase of acetylation of histone H3 tails (Fig. 3*B*).

MBD1 Associates with Methylated RARβ2 Promoter *in Vivo*. To investigate whether PML-RARα-mediated CpGs methylation creates docking sites for MBD1 at the endogenous RARβ2

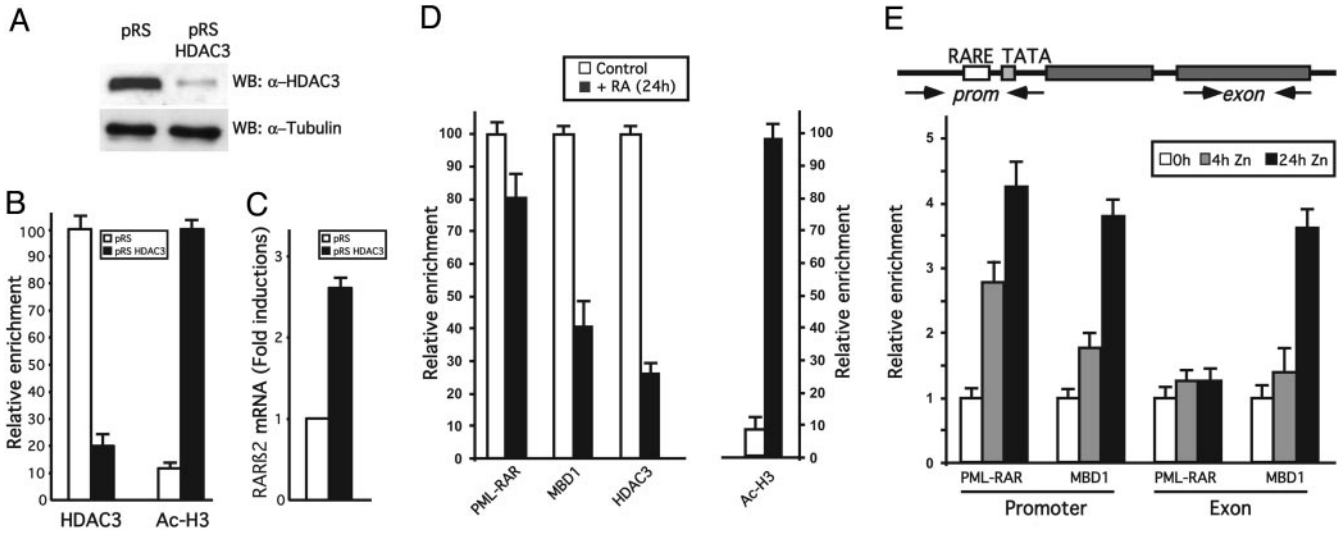

Fig. 3. Binding of PML-RARα and corepressors to the endogenous RARβ2 promoter. (*A*) Western blot analysis of total cell lysates derived from mock and HDAC3 interference RNA APL cells. Human NB4 leukemic cells were infected with a retroviral construct generating HDAC3-specific small hairpin RNA (pRS HDAC3) or the empty vector (pRS) and selected with puromycin for 3 days. Equal amounts of cell extract from mock and interference RNA cells were blotted with antibodies indicated. (*B*) Knock-down of HDAC3 in APL cells affects RARβ2 H3 acetylation levels. HDAC3 interference RNA cells (pRS HDAC3) or control cell (pRS) were subjected to ChIP analysis, as indicated. The promoter of RARβ2 was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments. (*C*) Knock-down of HDAC3 in APL cells affects RARβ2 promoter activity. Total RNA was prepared from cells as in *A*, and RARb2 gene expression was analyzed relative to GAPDH control by quantitative real-time PCR. Results are expressed as the mean ± SEM of two independent experiments performed in duplicate. (*D*) RA induces release of the PML-RARα corerepressor complex. NB4 cells were treated or not with RA (1 μM) for 24 h and then subjected to ChIP analysis, as indicated. The promoter of RARβ2 was amplified with real-time PCR. Error bars indicate the standard deviation obtained from three to five independent experiments. (*E*) PML-RARα recruits MBD1 to the RARβ2 5′ region. U937-PR9 cells were treated with Zn (0 h, 4 h, and 24 h) and then subjected to ChIP analysis. The promoter region and exon of RARβ2 were amplified with real-time PCR. Error bars indicate the standard deviation obtained from three independent experiments.

promoter, we performed chromatin immunoprecipitation (ChIP) experiments. In agreement with previous reports (9), PML-RARα was constitutively bound to the RARβ2 promoter sequence, regardless of the presence of RA (Figs. 3*D* and 8, which is published as supporting information on the PNAS web site). After equivalent ChIP analysis, MBD1 and HDAC3 were also found to be associated to RARβ2 promoter in untreated cells. In RA-treated cells, MBD1 and HDAC3 were substantially decreased in the promoter region, whereas histone H3 acetylation increased significantly (Fig. 3*D*). To study the kinetics of MBD1 recruitment to PML-RARα-methylated gene, we performed ChIP experiments in U937-PR9 cells, where PML-RARα expression is controlled by a Zn-inducible promoter (11). In these cells, PML-RARα expression leads to a time-dependent accumulation of mCpGs on the endogenous RARβ2 gene and to its transcriptional silencing. MBD1 was strongly associated with RARβ2 only in those cells in which PML-RARα had been expressed for 24 h (Figs. 3*E* and 7*B*). No enrichment was detected by using unrelated antibodies (data not shown). Interestingly, whereas PML-RARα binding was restricted to the promoter region harboring the RARE, MBD1 was also found outside the promoter (Fig. 3*E*).

Taken together, our results show a correlation among PML-RARα expression, promoter hypermethylation, and MBD1 occupancy of RARβ2 promoter/exon regions and suggest that PML-RARα (by means of DNMT recruitment) creates docking sites for MBD1 on its target promoter.

MBD1 Is Required for the Transforming Properties of PML-RARα. Because MBD1 synergizes with PML-RARα in promoter repression, we next wanted to investigate whether MBD1 likewise plays a role during the PML-RARα-induced differentiation block and whether MBD1 mutants could interfere with such a process. After Zn-induced expression of PML-RARα, U937-PR9 cells become refractory to VD/TGFβ differentiation stimuli, as measured by the expression of the CD14 differentiation marker (Fig. 4*A*, lane 2 versus lane 4). We cloned the wild type as well as the previously generated MBD1 mutants into a retroviral vector and used these to infect U937-PR9 cells. After selection, U937-PR9 cells were exposed to Zn for 16 h to induce PML-RARα expression with subsequent treatment with VD/TGFβ for 36 h. As expected, PML-RARα blocked differentiation of control cells (i.e., either not infected or infected with the empty viruses) by ≈70%. Overexpression of wild-type MBD1 further enhanced the ability of PML-RARα to prevent cell differentiation. Strikingly, overexpression of MBD1-dm (R22A/I527R) drastically inhibited the ability of PML-RARα to block hematopoietic differentiation as measured by FACS analysis of surface differentiation marker CD14 (Fig. 4*A*, lane 20). We also tested the consequence of the expression of MBD1 variants with single point mutation. Although all of these mutants reduced the PML-RARα block, they were not able to completely relieve it (Fig. 4*A*, lanes 12 and 16, and data not shown), similar to what was observed for promoter activity levels (Fig. 1*D*). Taken together, our data indicate that, in hematopoietic precursors, the association of MBD1 with methylated PML-RARα-target promoters is required to fully prevent cell differentiation. Expression of a mutated MBD1 that has lost the ability to bind DNA and to interact with HDAC3 interferes with the PML-RARα-induced differentiation block.

Discussion

In this report we show that MBD1 is required for silencing the PML-RARα target promoter RARβ2. After PML-RARα-induced promoter hypermethylation, MBD1 is recruited to and remains associated with the silenced RARβ2 promoter. Mutations in the MBD and TRDs of MBD1 restore RARβ2 transcriptional activity and prevent PML-RARα-induced hemato-

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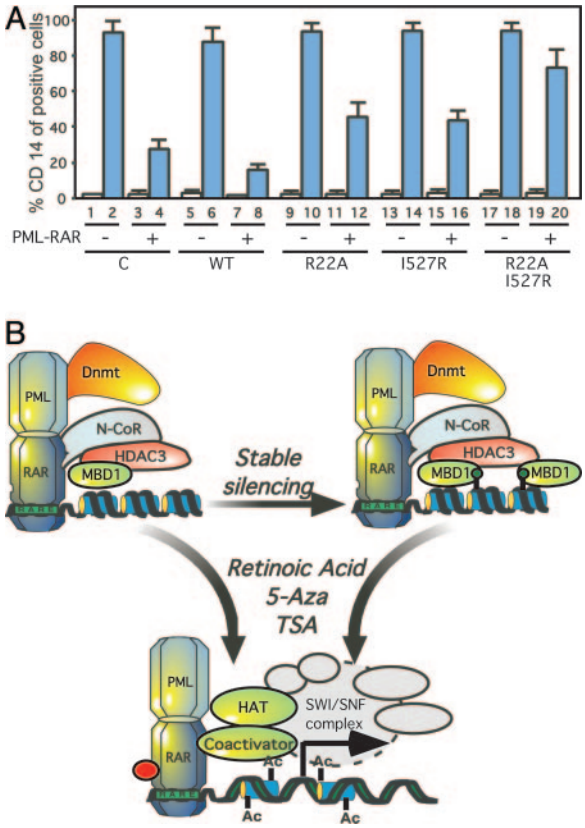

Fig. 4. MBD1 cooperates with PML-RARα-mediated block of hematopoietic differentiation. (*A*) U937-PR9 cells, after retroviral infection with MBD1 and MBD1 mutants or empty vector, were treated or not with Zn for 16 h, as indicated. Infected cells were treated either with vitamin D and TGFβ (blue bars) or with vehicle alone (ethanol, white bars). Cell differentiation was evaluated by quantitative expression of CD14 antigen (11). Error bars represent the standard deviation from the mean for triplicate experiments. (*B*) Model of promoter repression and activation mechanisms in leukemia. The oncoprotein PML-RARα binds to a well defined DNA sequence (5) and recruits NCoR, which in turn serves as platform for the interaction with HDAC3 and corepressors. The N-terminal region of HDAC3 is additionally responsible for the interaction with the TRD of MBD1. Similarly, PML establishes interaction with DNMTs (9). The activity of these corepressors leads to hypoacetylation of histone tails, DNA methylation (depicted by green lollipop), and transcriptional silencing. Methylated CpGs are potential docking sites for MBD1, which can in turn recruit further repressor enzymes. The progression wave of the proposed mechanism might ''close'' the chromatin structure and influence neighboring genes. Administration of RA, alone or in combination with TSA/5-Aza-dC, induces release of the corepressor complex and promotes recruiting of the coactivators containing histone acetyltransferases (HAT) and ATP-dependent chromatin remodeling activity (40).

poietic differentiation block. We provide evidence that HDAC3 is a common interactor for both PML-RARα and MBD1. APL cells knocked down for HDAC3 are impaired in PML-RAR-mediated gene silencing. Our findings demonstrate (*i*) a targeting mechanism for MBD recruitment by an oncogenic transcription factor, (*ii*) a direct role of MBD1 and HDAC3 in promoter silencing and in leukemia progression, and (*iii*) a time-dependent spreading of MBD1 occupancy outside of the promoter region.

MBD1 and Chromatin Alterations. Many human cancers are characterized by alterations in the balance of DNA methylation (18, 19). Our results indicate that stable binding of MBD1 to the RARβ2 promoter occurs 24 h after PML-RARα induction, concurrent with the CpG methylation within the promoter region and exons. At an earlier time point (4 h) in the absence of CpG methylation, the association of MBD1 with RARβ2 is confined to the PML-RARα binding region, thus suggesting a direct recruitment by means of the oncoprotein. The association between MBD1 and PML-RARα could be essential to increase the ''local'' concentration of effectors proteins, thus increasing the probability of efficient binding of MBD1 to methylated CpGs. Similar scenarios have been postulated for the Rb/HDAC/Suv39H1/HP1 complex (20) and for the assembly of the RNA polymerase I complex on ribosomal genes (21). Because DNA methylation is often altered in cancer, and because MBD proteins are the functional interpreters of DNA methylation, a crucial role for MBD proteins in cancer can be postulated.

Previously, it was shown that neither HDAC1 nor HDAC2 is responsible for MBD1-mediated repression (15). Here we present evidence that the histone deacetylation-dependent repressor property of MBD1 is due to its interaction with HDAC3. The TRD of MBD1 and the N-terminal region of HDAC3 mediate this interaction. Several groups have recently demonstrated that MBD proteins (including MBD1) can establish interactions with several histone methyltransferases (22, 23) and DNMTs (24). Given the network of interactions among these factors, one could envision a model whereby binding of MBD1 plays a pivotal role in both establishing and maintaining epigenetic modification across the RARβ2 locus, with PML-RARα being the ''initiator'' factor.

PML-RARα-Mediated Repression. The recruitment of HDAC3 by PML-RARα is of particular interest because it is found in a tight complex with the nuclear corepressor SMRT/N-CoR (25–28) and is critical for repression by multiple transcription factors (29–31). Despite the fact that other HDACs are present in the corepressor complex, the HDAC activity of the complex as well as its integrity depend completely on the presence of HDAC3 as well as its association with SMRT/N-CoR (32). Thus, the RAR moiety of PML-RARα, through direct interactions with SMRT/N-CoR (10, 11), likewise recruits a multiprotein corepressor complex to its target genes, whereas the PML moiety mediates the interactions with DNMTs. These data establish a direct connection between DNA methylation and histone deacetylation in leukemia and further support the concept of interdependent processes between these two layers of epigenetic control (Fig. 4*B*). At pharmacological doses, RA overcomes this repression and induces epigenetic modifications at its target loci through a coordinated down-regulation of cellular DNMT expression (33) and specific recruitment of coactivators at RARE-containing genes, such as RARβ2 (Fig. 4*B*). Under this condition, MBD1 dissociates from target promoters, which is reminiscent of the dynamic association displayed by MeCP2 to the *BDNF* and *Hairy2a* genes (34–36). However, clinical evidence indicates that RA *per se* is unable to eradicate the leukemic clone and to cure this disease. Thus, understanding the molecular mechanism of gene silencing is important for developing new antileukemic strategies. In the present study we have demonstrated that MBD1 mutated in both the MBD and TRDs acted in a dominant-negative manner (Figs. 2*B* and 4*A*), because it impaired the ability of PML-RARα to block hematopoietic cell differentiation. Our data suggest that MBD1 forms oligomers in the cell nucleus, which would explain its dominant-negative phenotype, because this mutant could sequester wild-type MBD1 into nonfunctional complexes (R.V. and L.D.C., unpublished data). Because MBD1 is located at the core of the chromatin structure of methylated DNA regions, the dissociation of MBD1 (or the prevention of its interaction with HDACs) might stimulate chromatin remodeling and further release the molecules packed into the chromatin. Further characterization of the PML-RARα–corepressor complex, which establishes and allows spreading of the silenced state, will provide insight into

crosstalk among the different epigenetic layers as well as into the molecular pathology of leukemia.

Materials and Methods

Plasmids and Oligonucleotides. The plasmid pMBD1GFP was kindly provided by A. Bird. (Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh). MBD1 mutants were made by introducing point mutations into pMBD1GFP vector by using the Stratagene mutagenesis kit. Retroviral expression vectors were generated by subcloning cDNA of all forms of MBD1 into PINCO (37). The expression vector for PML-RARα, the RARβ2-luc reporter plasmid, and the CMV-β-galactosidase were described in ref. 9. pRS-HDAC3 was generated by ligating synthetic oligonucleotides against the target sequence into pRETRO-SUPER. Flag-HDAC3 and GST-HDAC3 deletion mutants were kindly provided by E. Seto (H. Lee Moffitt Cancer Center at the University of South Florida, Tampa) (38). Primer sequences and PCR conditions are available upon request.

Cell Lines, Transfection, and Retroviral Infection. HEK 293T and HeLa cells were transfected by standard methods. As an internal reference for transfection efficiency, 20 ng of the pCMVβ-Gal plasmid were also cotransfected. Cell extracts were prepared as described in ref. 9. PINCO-based and pRS-based retroviruses were produced by transfected Phoenix packaging cells. The collected retrovirus was subsequently used to infect NB4 and U937-PR9 hematopoietic precursor cells.

Immunoprecipitation and ChIP. For immunoprecipitations, antibodies were coupled to protein A Sepharose beads. Cell extracts were prepared in lysis buffer (50 mM Hepes, pH 7.5/150 mM NaCl/1 mM EDTA/2.5 mM EGTA/0.1% Tween 20/1 mM phenylmethylsulfonyl fluoride/0.4 units/ml aprotinin and leupeptin/1 mM NaF/0.1 mM $NaVO_4$) and incubated with beads for 8 h at 4°C. Beads were washed five times with lysis buffer complemented with additional 150 mM NaCl and 0.1% Nonidet P-40. Bound proteins were eluted with 2× Laemmli sample buffer and loaded on SDS/PAGE. Input lanes show 5% of lysates used for precipitation. Antibodies were described previously (PGM3, ref. 9) or are commercially available (anti-MBD1 from Imgenex, anti-FLAG from Sigma, and anti-HDAC3 from Abcam).

For ChIP, NB4 or U937-PR9 cells were crosslinked with 1% formaldehyde (Sigma) at 37°C for 10 min. Cells were rinsed twice with ice-cold PBS and collected. ChIPs were performed and analyzed as described in ref. 39. The immunoprecipitated DNA was quantified by real-time quantitative PCR (Roche LightCycler). The sequences of the PCR primers are available upon request.

Cell Differentiation. Differentiation of U937-PR9 cells under the influence of dihydroxyvitamin D_3 (250 ng/ml) and TGFβ (1 ng/ml) was performed as described in ref. 9. The percentage of differentiated antigen-positive cells and the fluorescence were analyzed by flow cytometry on Becton Dickinson FACScan with appropriate antibodies, such as CD14 (BD Biosciences).

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