MBD3, a Component of the NuRD Complex, Facilitates Chromatin Alteration and Deposition of Epigenetic Marks

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In plants, as in mammals, mutations in SNF2-like DNA helicases/ATPases were shown to affect not only chromatin structure but also global methylation patterns, suggesting a potential functional link between chromatin structure and epigenetic marks. The SNF2-like ATPase containing nucleosome remodeling and deacetylase corepressor complex (NuRD) is involved in gene transcriptional repression and chromatin remodeling. We have previously shown that the leukemogenic protein PML-RARα represses target genes through recruitment of DNA methyltransferases and Polycomb complex. Here, we demonstrate a direct role of the NuRD complex in aberrant gene repression and transmission of epigenetic repressive marks in acute promyelocytic leukemia (APL). We show that PML-RARα binds and recruits NuRD to target genes, including to the tumor-suppressor gene RARβ2. In turn, the NuRD complex facilitates Polycomb binding and histone methylation at lysine 27. Retinoic acid treatment, which is often used for patients at the early phase of the disease, reduced the promoter occupancy of the NuRD complex. Knockdown of the NuRD complex in leukemic cells not only prevented histone deacetylation and chromatin compaction but also impaired DNA and histone methylation, as well as stable silencing, thus favoring cellular differentiation. These results unveil an important role for NuRD in the establishment of altered epigenetic marks in APL, demonstrating an essential link between chromatin structure and epigenetics in leukemogenesis that could be exploited for therapeutic intervention.

The product of the chimeric gene generated by the 15;17 chromosome translocation, PML-RARα, is a well-characterized oncogenic transcription factor found in the majority of human acute promyelocytic leukemias (APL) (8). Ectopic expression of the fusion protein in hematopoietic precursor cells blocks differentiation and promotes leukemia development (26). The oncogenic potential of PML-RARα is based on the aberrant silencing of genes, including several tumor suppressor genes. PML-RARα, like the wild-type form of retinoic acid receptor (RAR), represses transcription of target genes through binding to so-called RA responsive elements (RARE) and subsequent recruitment of corepressors such as histone deacetylases (15, 22). In contrast to the wild-type RARα, the fusion protein PML-RARα is rendered insensitive to physiological concentrations of RA that would usually trigger transcriptional activation and therefore functions as a constitutive and potent transcriptional repressor of RARE-containing promoters. However, pharmacological doses of RA (1 μM), which are used for patients at the early phase of the disease, can lead to partial derepression of PML-RARα target genes (27). We have demonstrated that the transcriptional repression of PML-RARα target genes is further reinforced by recruitment of Polycomb complex (32) and DNA methyltransferases (DNMTs) (9, 31). Once established, the PML-RARα-induced epigenetic modifications and chromatin changes are stable and maintained throughout cell divisions.

Recently, an interesting functional link between chromatin structure and DNA methylation has been proposed (1): in plants, as in mammals, mutations in SNF2-like DNA helicases/ATPases were shown to affect not only chromatin structure but also global methylation patterns.

In the past few years, several DNA helicase/ATPase-containing complexes have been characterized that facilitate or repress transcription by utilizing the energy of ATP to alter the histone-DNA interface within the nucleosome structure (35). Among these, the nucleosome remodeling and deacetylase corepressor complex (NuRD) contains at least seven polypeptides, including histone deacetylase 1 (HDAC1) and HDAC2, H4 interacting proteins (RbAp46/48), methyl-binding protein 3 (MBD3), MTA-family members (MTA1 to MTA3) (12), and an SNF2-like chromatin-remodeling ATPase (Mi-2/CHD4) (7, 29, 34, 37, 39). Genetic and molecular data suggest that MBD3 is important for the integrity and stability of the NuRD complex (16), and it is implicated in the regulation of mouse embryonic stem cell pluripotency and self-renewal (19). The recruitment of the NuRD complex to DNA can occur through interaction with MBD2 (11) or with several sequence-specific DNA-binding proteins (12, 21, 23, 28).

We demonstrate here that NuRD plays an important role in the hematopoietic differentiation block induced by PML-RARα expression. We show that PML-RARα binds and recruits NuRD to target genes, which in turn leads to chromatin compaction. Furthermore, binding of NuRD at target genes allows recruitment of the Polycomb repressive complex 2
(PRC2) and DNMT3a, with consequent promoter silencing. Retinoic acid (RA) treatment, which is often used for patients at the early stage of the disease, reduced the occupancy of the NuRD complex at target genes. Together, these results unveil a novel function for the NuRD complex in leukemogenesis, and establish a link between NuRD activity and epigenetic alterations in cancer.

MATERIALS AND METHODS
Plasmids. The FlaghMBD3a vector was kindly provided by F. Ishikawa (Kyoto University), pGEX-5X-3 mMBD3a and pGEX-5X-3 mMBD3b, antibodies against MBD3 were kindly provided by D. Reinberg (University of Medicine and Dentistry of New Jersey), pCneo-mHDAC1-Myc and pME18s-Flag-mHDAC2 were kindly provided by C. Seiser (University of Vienna), and pGEX-MTA1/2/3 and pcDNA-Flag-MTA1/2/3 were kindly provided by W. M. Yang (National Cheng Hsing University).

HA mMBD3b was subcloned from pGEX-5X-3 mMBD3b to pcDNA3 by digestion with EcoRV and Xhol enzymes.

Cell lines and transfections. NB4 cells and U937-P9 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 Dulbecco modified Eagle medium supplemented with 10% of fetal bovine serum and then transfected by using the calcium phosphate coprecipitation method.

Immunoprecipitation and chromatin immunoprecipitation. For immunoprecipitation, 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, 500 ng of protamine sulfate (5 g/ml), and viruses containing the shMBD3, shSuz12, or shScramble. After 36 h, infected cells were selected with puromycin (2 μg/ml for U937-P9) or 1 μg/ml for NB4) for at least 72 h.

For rescue of phenotype experiments, MBD3 from the pcDNA-Flag-MBD3 plasmid was mutated in bp 10 of the MBD3 short hairpin binding site by using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Mutated MBD3 (MBD3-rescue) was cloned into the retroviral pMSCV2.2-green fluorescent protein (GFP) plasmid.

Then, 8 μg of pMSCV2.2-MBD3-T369A-GFP and pMSCV2.2-GFP vectors were transfected with 4 μg of the pSVSVG plasmid by the calcium phosphate precipitation method into HEK-293T/GFP cells. After 2 and 3 days, supernatants containing retroviruses were collected. U937-P9 cells were spin infected for 90 min (3,200 rpm) in the presence of Polybrene (10 μg/ml) and viruses containing shM2A2, shM2-2, or shScramble. After 36 h, the cells were GFP sorted. The RNA purification and reverse transcription-PCR (RT-PCR) analysis. From U937-P9 MBD3 RNA interference cells (RNAi MBD3) or from U937-P9 control cells (RNAi control) after no treatment, after RA treatment (1 nM, 36 h), and after RA treatment (1 nM, 12 h) with subsequent Zn induction (100 mM Zn, 24 h) was extracted by using a Qiagen RNeasy minikit, retrotranscripted (avian myeloblastosis virus; Roche), and assayed for the expression of RARβ2 by using quantitative real-time PCR (Roche LightCycler). The sequences of the PCR primers are available upon request.

RESULTS
NuRD knockdown in leukemic cells. It has been proposed that chromatin remodeling machineries might contribute to the establishment and/or maintenance of epigenetic marks. We and others have previously demonstrated that PML-RARα imposes an altered pattern of DNA methylation (9) and lysine 27 methylation (32) at its target genes. We hypothesized that NuRD could be implicated in PML-RARα-induced gene silencing since (i) one of the HDACs enzymes present in the complex has been demonstrated to associate with the PML moiety of PML-RARα (36); (ii) NuRD complex is mainly involved in gene repression, while other chromatin remodeling complexes (e.g., SWI/SNF) are either exclusively involved in gene activation or function in both activation and repression; and (iii) NuRD has been implicated in hormone receptor-mediated gene silencing (37). We thus decided to investigate whether the NuRD complex would be involved in the maintenance of the cancerous phenotype in fully established patient-derived NB4 leukemic cells. It has been previously shown that reduction of MBD3 protein levels leads to the disassembly of the NuRD complex concomitantly with decreased intracellular levels of some of other subunits (16, 19). Therefore, in order to...
FIG. 1. NuRD knockdown in APL facilitates differentiation. (A) Western blots analysis of mock and MBD3 interference NB4 cells. Equal amounts of cell extracts from mock (RNAi-Scr) and RNAi cells (RNAi-MBD3) were blotted with the indicated antibodies. (B) Differentiation of...
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We have previously demonstrated that the leukemic potential of PML-RARα relies—at least in part—on its ability to induce epigenetic alterations at target genes (17), which include DNA methylation (9) and trimethylation of lysine 27 of histone H3 (H3K27me3) (32), which is catalyzed by the Polycomb repressive complex 2 (PRC2). Thus, based on the results described above, we hypothesized that MBD3 knockdown could affect DNA and histone methylation at PML-RARα-repressed genes. Among several PML-RARα target genes, RARβ2 has been characterized in great detail (reference 10 and reference therein). The genomic bisulfite analysis confirmed that in control cells the CpG dinucleotide in each sequenced cloned is depicted by a red square if the position was methylated or a white square if it was not. (D) NuRD knockdown affects H3 methylation at lysine 27 (H3K27me3). ChIP assays were performed in mock (RNAi-Scr) and RNAi-MBD3 NB4 cells as described in Fig. 2A. The promoter of RARβ2 was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (E and F) Western blots and differentiation (NBT) analysis of mock and MTA2 interference NB4 cells. (G and I) Western blots, differentiation (NBT), and DNA methylation analysis of mock and MBD3 interference HL60 cells.

mock and RNAi-MBD3 NB4 cells into granulocytes. Cells were stained with Wright-Giemsa and analyzed for the morphology under the light microscopy. Differentiation was evaluated by NBT reduction assay. (C) NuRD knockdown affects the DNA methylation levels at the RARβ2 gene. DNA extracted from mock (RNAi-Scr) and RNAi-MBD3 NB4 cells was used for bisulfite genomic sequencing. The methylation status of each CpG dinucleotide in each sequenced cloned is depicted by a red square if the position was methylated or a white square if it was not. (D) NuRD knockdown affects H3 methylation at lysine 27 (H3K27me3). ChIP assays were performed in mock (RNAi-Scr) and RNAi-MBD3 NB4 cells as described in Fig. 2A. The promoter of RARβ2 was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (E and F) Western blots and differentiation (NBT) analysis of mock and MTA2 interference NB4 cells. (G and I) Western blots, differentiation (NBT), and DNA methylation analysis of mock and MBD3 interference HL60 cells.

ablate NuRD activity, we generated a stable MBD3 knockdown in NB4 cells (RNAi MBD3), using a retroviral vector-based shRNA approach, with strongly reduced expression of MBD3 (more than 70%, Fig. 1A). Under this experimental condition, the levels of MTA2 proteins were also reduced, while the levels of HDAC enzymes were not affected (Fig. 1A and data not shown). Importantly, coinmunoprecipitation analysis confirmed that the NuRD complex was not formed, as also previously described (16, 19; data not shown). Next, we assessed differentiation based on morphological and functional criteria. NB4 RNAi control and MBD3 RNAi cells were stained with Wright-Giemsa (Fig. 1B, left panel). As shown, MBD3 RNAi cells displayed a more mature morphology, as observed by several features: (i) they contained a reduced nucleus/cytoplasm ratio, (ii) the dark blue-gray cytoplasm became lighter and often contained granules, and (iii) they frequently displayed an irregular nucleus shape. Furthermore, the NBT reduction test, which measures functional differentiation, was performed using standard methodology. The percentage of cells containing intracellular-reduced black formazan deposits was determined. While we observed only a modest amount of cells containing intracellular-reduced black formazan deposits was determined. While we observed only a modest amount of spontaneous differentiated cells in control samples, we observed a significantly increased number of NBT-positive cells in RNAi MBD3 NB4 (Fig. 1B, right panel). Together, these results show that MBD3 protein is required to fully maintain its was determined. While we observed only a modest amount of spontaneous differentiated cells in control samples, we observed a significantly increased number of NBT-positive cells in RNAi MBD3 NB4 (Fig. 1B, right panel). Together, these results show that MBD3 protein is required to fully maintain the undifferentiated and leukemic phenotype of NB4 cells.

Epigenetic changes in NuRD knockdown cells. We have previously demonstrated that the leukemic potential of PML-RARα relies—at least in part—on its ability to induce epigenetic alterations at target genes (17), which include DNA methylation (9) and trimethylation of lysine 27 of histone H3 (H3K27me3) (32), which is catalyzed by the Polycomb repressive complex 2 (PRC2). Thus, based on the results described above, we hypothesized that MBD3 knockdown could affect DNA and histone methylation at PML-RARα-repressed genes. Among several PML-RARα target genes, RARβ2 has been characterized in great detail (reference 10 and reference therein). The genomic bisulfite analysis confirmed that in control cells the RARβ2 promoter is hypermethylated to a similar extent as previously reported, while in cells lacking functional NuRD complex the levels of CpG methylation were significantly reduced (Fig. 1C). In parallel with the previous analysis, we next performed ChIP experiments for the H3K27me3. A three- to fourfold reduction in H3K27me3 was observed in MBD3 knockdown cells compared to the mock control (RNAi control) (Fig. 1D). Similarly, promoter methylation was significantly reduced in RNAi MTA2 (Fig. 1E and data not shown), while an increased number of NBT-positive cells were observed in RNAi MTA2 NB4 compared to control cells (Fig. 1F).

As a further control for the specificity of our results, we generated a new stable MBD3 knockdown in acute myeloid leukemia (AML) HL60 cells (Fig. 1G), which are derived from an AML patient and are negative for PML-RARα but positive for wild-type RARαs. We thus assessed differentiation based on morphological and functional criteria. No differences between HL60 control RNAi and MBD3 RNAi cells were observed for differentiation-associated antigens, morphology, or NBT reduction (Fig. 1H and data not shown). We then analyzed by bisulfite genomic sequencing the methylation status of the RARβ2 promoter in HL60 cells with or without MBD3. The level of methylated CpGs in control HL60 cells is similar to that observed in NB4 cells; however, knockdown of MBD3 does not cause any decrease of DNA methylation in HL60 cells (Fig. 1I), in contrast to the 70% reduction observed in NB4 MBD3 RNAi (Fig. 1G).

These results revealed an unexpected role of the NuRD complex in the metabolism of epigenetic marks in leukemic cells and prompted us to further investigate not only whether NuRD is present at target promoters but also whether it participates in initiating epigenetic alterations in PML-RARα-expressing cells.

RA reduces NuRD occupancy at PML-RARα target genes. In order to explore the possibility of NuRD occupancy at PML-RARα target genes, we performed ChIP experiments in NB4 cells. Several NuRD proteins were found associated at the RARE region of the endogenous RARβ2 promoter (Fig. 2A) in untreated cells, while a pharmacological dose of RA (1 μM) caused a reduction of the NuRD complex, with a corresponding increase of histone acetyltransferases p300 and CBP and of histone H3 acetylation levels (Fig. 2A). Similarly, the promoter occupancy of Brahma-related gene 1 (BRG1), a subunit of the SWI/SNF chromatin remodeling complex, as well as polymerase II, was increased upon RA treatment, resulting in promoter reactivation (Fig. 2B). Importantly, similar analysis was performed at the pS2 promoter, a well-characterized estrogen-induced gene that is insensitive to RA stimulation (25, 30). None of the NuRD subunit was found associated at the pS2 promoter (data not shown). To strengthen our observations, we expanded our analysis to other PML-RARα target genes (24). Among these, 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 RARβ2, promoters of these other target genes showed a similar reduction of MTA2 and concomitantly an increase of H3ac upon RA administration (Fig. 2C).

Targeting NuRD complex at PML-RARα target genes. We hypothesized that PML-RARα recruits the NuRD complex to its target genes. We performed ChIP experiments in the hematopoietic precursor cells U937-PR9. In contrast to NB4, which constitutively express PML-RARα, U937-PR9 cells con...
FIG. 2. Binding of PML-RARs and NuRD complex to the endogenous RARβ2 promoter is RA sensitive. (A) In NB4 cells, the NuRD complex is bound to the RARβ2 promoter and it is released after 8 h of RA (1 μM). NB4 cells were subjected to ChIP analysis as indicated in the figure. The RARβ2 promoter was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (B) Endogenous RARβ2 expression is upregulated after 8 h of RA (1 μM) treatment. Semiquantitative RT-PCR of cDNA from 1 μg of RNA. Tubulin expression levels were used as a PCR control. (C) The NuRD complex is released from NFE2 and PSCD4 promoters after pharmacological RA treatment. A ChIP assay was performed as in panel A. Errors bars indicate the standard deviation obtained from three independent experiments. (D) PML-RARs recruits the NuRD complex to the RARβ2 promoter. U937-PR9 cells, treated sequentially with RA (1 nM) to activate endogenous RARs and then with 100 μM Zn (12 h) to induce PML-RARs expression, were subjected to ChIP analysis, as indicated in the figure. The RARβ2 promoter was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments.
tain a zinc-inducible promoter controlling PML-RARα expression, making them an ideal cellular system for studying the early epigenetic events associated with the oncogene expression. PML-RARα binding to RARβ2 promoter was restricted to the promoter region harboring the RARE (Fig. 2D) (31). Upon expression of PML-RARα, HDAC2 and MTA2 were enriched at endogenous target genes, while the occupancy of histone acetyltransferase enzyme p300 and histone H3 acetylation was reduced (Fig. 2D and 5B).

Increased occupancy of NuRD complex at target genes could be due to its direct targeting. We thus investigated whether PML-RARα interacts with the NuRD remodeling complex. We found that both MBD3a and MBD3b isoforms associated with the oncprotein when co-overexpressed in 293T cells (Fig. 3A). We then extended our analysis to other components of the NuRD complex. PML-RARα interacted with MTA2 and to a lesser extent with MTA1 and MTA3. Finally, both HDAC1 and HDAC2 associated with PML-RARα (data not shown).

To investigate whether PML-RARα associates with physiological levels of the NuRD complex, we performed coimmunoprecipitation experiments using lysates from the two best-characterized APL model systems: patient-derived NB4 and U937-PR9 cells. We consistently found that in the same coimmunoprecipitated material, PML-RARα associated with all of the NuRD components, including MTA2, Mi-2, and HDAC1/2.
FIG. 4. The NuRD complex contributes to RARβ2 promoter epigenetic silencing. (A) NuRD complex is necessary for the complete repression of the RARβ2 gene induced by PML-RARα. U937-PR9 cells were treated with physiological concentration of RA (1 nM). mRNA levels of endogenous RARβ2 gene were measured by real-time PCR. The results were normalized against β-actin mRNA levels. (B) Western blots analysis of mock (RNAi-Scr) and MBD3 interference U937-PR9 cells (RNAi-MBD3). Equal amounts of cell extract from mock and RNAi-MBD3 cells were blotted with the indicated antibodies. (C) NuRD facilitates DNMT3a binding at RARβ2 genes. ChIP assays were performed in mock
(Fig. 3B and C). The interaction resisted the presence of ethidium bromide in the precipitation reaction (Fig. 3D), as well as a high-salt extraction method (data not shown), thus excluding the possibility of a DNA-mediated protein association, suggesting a direct interaction between PML-RARa and the NuRD complex. RA induces structural change in PML-RARa that dissociates all of the corepressors binding and increases the affinity for the binding of coactivators (31). Interestingly, the interaction between PML-RARa and NuRD was dramatically reduced in the presence of pharmacological doses of RA (Fig. 3C).

MBD3 is required for epigenetic silencing of PML-RARa target promoters. We next wanted to investigate whether MBD3 is required for PML-RARa-mediated gene silencing. First, we tested the ability of PML-RARa to downregulate its target gene RARb2 in U937-PR9 cells when stimulated with physiological concentration of RA (1 nM) (Fig. 4A, lane 3 versus lane 7). We then generated a stable MBD3 knockdown in U937-PR9 cells (RNAi MBD3). A reduction of >75% of MBD3 protein was achieved under these conditions (Fig. 4B). Similarly to NB4 cells, in U937-PR9 cells the knockdown of MBD3 led to a reduction of intracellular MTA2, but it did not affect HDAC1/2 and DNMTs (Fig. 4B and data not shown). In contrast to control cells, in RNAi MBD3 cells the RARb2 promoter was not efficiently silenced by PML-RARa (Fig. 4A, lanes 3 and 4 versus lanes 7 and 8).

We and others have previously demonstrated that PML-RARa and other transcription factors can repress transcription by recruitment of DNMTs (e.g., STAT5, AML1-ETO, and Myc) (2, 38), leading to promoter hypermethylation. We next analyzed the occupancy of DNMTs at RARb2 promoter in both RNAi control cells and in RNAi MBD3 cells. As shown in Fig. 4C, the accumulation of DNMT3a, which was observed in the presence of PML-RARa, was significantly reduced in RNAi MBD3 cells. We did not observe any variation in DNMT1 occupancy. Previously, DNMT3a has been reported to be able to bind MBD3 (5). In addition, we found that HDAC1 and the NuRD-specific subunit MTA2 bind to in vitro-translated DNMT3a (data not shown). These results prompted us to investigate the status of methylation of the RARb2 promoter in dependence of PML-RARa and NuRD. Thus, we performed bisulfite genomic sequencing analysis in the same experimental setting as described above. As previously reported, the expression of PML-RARa caused RARb2 promoter hypermethylation in wild-type or mock-infected leukemic cells (Fig. 4D and data not shown). Interestingly, in MBD3 knockdown cells the methylation of CpGs was reduced by 50%. Of note, the protein levels of DNMT3a and DNMT1, as well as the global methyltransferase activity, were not altered by RNAi MBD3 (data not shown).

We next investigated whether Mi2, the ATPase subunit of the NuRD complex, is necessary for DNA methylation of PML-RARα-target genes. We thus generated a stable Mi2 knockdown in U937-PR9 cells (RNAi-Mi2; Fig. 4E) and performed a bisulfite genomic sequencing of RARb2 promoter using the RNAi-Scr and RNAi-Mi2 cell lines. We observed that in Mi2 knockdown cells, PML-RAR-induced DNA methylation was reduced by more than 50% (Fig. 4D). Similarly, promoter methylation was significantly reduced in RNAi MTA2 (Fig. 4D and F), corroborating our conclusion that the NuRD complex and its associated enzymatic activities play an important role in PML-RARa-induced DNA methylation.

Role of the NuRD complex in the cross talk between DNA and histone methylation. The results described above suggest that the NuRD complex is required for PML-RARa-induced de novo DNA methylation. We recently demonstrated that the presence of DNA methylation and Polycomb are both necessary for maintenance of the epigenetic alterations in leukemic cells that constitutively express PML-RARa (32). We thus decided to investigate how epigenetic silencing is initiated following the expression of the oncoprotein PML-RARa. For this, we analyzed not only the kinetics of NuRD, DNMTs, and Polycomb recruitment but also their interdependence for the establishment of the epigenetic repressive marks at promoters.

Time course ChIP analysis performed in U937-PR9 cells indicated that the NuRD complex is efficiently loaded at RARb2 promoter after only 8 h of expression of PML-RARa, as measured by the presence of the diagnostic subunit MTA2 (Fig. 5A). Similar analyses suggested that EZH2 and DNMT3 are found significantly associated at the promoter region at later time points, with DNMT3 being strongly recruited at 48 h. Interestingly, in cells lacking active NuRD complex, PML-RARa-mediated EZH2 recruitment and H3K27me3 were severely compromised (Fig. 5B), while the global H3 acetylation level was higher.

The specificity of our observations was further investigated by reintroducing a Mi2 silent mutant (3), which is insensitive to the shRNAs (Fig. 5C), into MBD3 knockdown cells. To this end, we generated stable cell lines wherein the NuRD rescue vector (MBD3-rescue) or a control vector was infected into RNAi-MBD3 cells. GFP-positive infected cells were then sorted, and MBD3 protein levels were analyzed by Western blotting (Fig. 5D, left panel). Interestingly, the interaction between MTA2 and HDAC1 was restored in MBD3-rescued cells (Fig. 5D, right panel). In addition, aberrant H3K27 methylation and gene silencing was also observed upon PML-RARa expression (Fig. 5E and F). We conclude that the observed effects of the shRNA-Mi2 are due to specific effects on the intended target and cannot be explained by off-target effects. Similarly, knockdown of the Mi2 or MTA2 subunit of the NuRD complex (Fig. 4E and F) impaired deposition of the H3K27 methylation marks (Fig. 5G and H). We next explored whether knockdown of the Polycomb repressive complex 2

(RNAi-Scr) and RNAi-MBD3 U937-PR9 cells as described in Fig. 2A. The promoter of RARβ2 was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (D) NuRD knockdown reduces DNA methylation of RARβ2 gene. DNA extracted from mock (RNAi-Scr), RNAi-MBD3, RNAi-Mi2, and RNAi-MTA2 U937-PR9 cells untreated or treated with 100 μM Zn for 24 h was used for bisulfite genomic sequencing. The methylation status of each CpG dinucleotide in each sequenced cloned is depicted by a red square if the position was methylated or a white square if it was not. (E and F) Western blots analysis of mock, Mi2, and MTA2 interference U937-PR9 cells. Equal amounts of cell extracts from mock (RNAi-Scr) and RNAi cells were blotted with the indicated antibodies.
FIG. 5. DNA and histone methylation dependency in APL leukemic cells. (A) Kinetics of NuRD, EZH2, and Dnmt3a/b recruitment to RARα/H9252 promoter. U937-PR9 cells, treated with 100 μM Zn for 8, 24, and 48 h to induce PML-RARα expression, were subjected to ChIP analysis as indicated in the figure. The RARα/H9252 promoter was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (B) NuRD complex is required for PML-RARα-mediated Polycomb recruitment and H3K27me3 at RARα/H9252 promoter. ChIP assays were performed in mock (RNAi-Scr) and RNAi-MBD3 U937-PR9 cells, as described in Fig. 2A. The promoter of RARβ2 was
(PRC2) would also affect NuRD occupancy and gain of DNA methylation at RARβ2 promoter. We generated a stable SUZ12 knockdown U937-PR9 cell line (RNAi SUZ12). A reduction of >70% of SUZ12 protein was achieved under these conditions compared to the mock knockdown cells (RNAi control) (Fig. 6A), and a corresponding decrease in bulk histone H3 tri-methyl K27 level was also observed. ChIP analysis for MTA2 indicates that the PRC2 complex is not amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments. (C) Schematic representation of the silent mutation in MBD3. (D) A silent mutant of MBD3 rescues interaction between NuRD components. Equal amounts of cell extract from mock, MBD3 interference (RNAi-MBD3), and MBD3-rescue U937-PR9 cells untreated or treated with 100 μM Zn for 24 h was used for bisulphite genomic sequencing. The methylation status of each CpG dinucleotide in each sequenced cloned is depicted by a red square if the position was methylated or a white square if it was not.

FIG. 6. NuRD occupancy at RARβ2 promoter is independent of PRC2 complex. (A) Western blot analysis of mock and Suz12 interference U937-PR9 cells. Equal amounts of cell extract from mock and RNAi-MBD3 cells were blotted with the indicated antibodies (left panel). ChIP analysis was performed using antibodies indicated in the figure. The RARβ2 promoter was amplified by real-time PCR. Errors bars indicate the standard deviation obtained from three independent experiments (right panel). (B) Polycomb knockdown reduces DNA methylation of the RARβ2 gene. DNA extracted from mock (RNAi-Scr) and RNAi-Suz12 U937-PR9 cells untreated or treated with 100 μM Zn for 24 h was used for bisulphite genomic sequencing. The methylation status of each CpG dinucleotide in each sequenced cloned is depicted by a red square if the position was methylated or a white square if it was not.

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required for PML-RARa-mediated NuRD recruitment, although EZH2 binding and H3K27 trimethylation were found to be reduced. Interestingly, in cells lacking PRC2 complex, DNA methylation fails to accumulate at the RARb2 promoter upon PML-RARa expression, while in RNAi control cells, PML-RARa caused promoter hypermethylation (Fig. 6B). Together, these experiments suggest that the NuRD complex is required for the establishment of DNA and histone methylation at lysine 27, while PRC2 likely plays a role in sustaining active DNA hypermethylation (Fig. 7).

DISCUSSION

The intimate link between NuRD and DNA methylation was postulated since the identification of MBD2 and MBD3 within the MeCP1/NuRD complex. It was later demonstrated that, although MBD2 is not an integral subunit of NuRD, it associates and recruits the NuRD complex to methylated DNA. More recently, Feng and Zhang provided additional mechanistic insight on the MBD2-dependent binding of NuRD to methylated nucleosomes (11). Our data now reveal a novel function of NuRD in this scenario. We found that the establishment of DNA methylation patterns at PML-RARa target genes is altered in cells lacking the NuRD complex. Based on this finding we speculate that the remodeling activity of NuRD may facilitate access of DNMTs to chromatin template for deposition of methyl groups at CpG sites. Alternatively, a continuous binding of NuRD to silenced promoters could be required for the maintenance (and perpetuation during cell cycle) of methylated CpGs. It would be interesting to investigate in the future whether DNA methylation levels of other genes, which are not PML-RARa direct targets, are also affected in cells lacking NuRD complex and more in general how chromatin structure influences DNA methylation pattern. Consistent with this hypothesis, the chromatin remodeling SNF2-like protein DDM1 was shown to be essential for full methylation of the Arabidopsis thaliana genome (33). In mammals, mutations in mouse Lsh genes (6) and human ATRX (13, 14), both of which encode relatives of the chromatin-remodeling protein SNF2, have significant effects on global DNA methylation patterns. Thus, both global and targeted DNA methylation might require a dedicated chromatin remodeling machinery. The aforementioned link between NuRD and MBDs, as well as the interaction between NuRD and DNMTs documented here, identify NuRD as an important player in DNA methylation metabolism in tumor cells and likely in normal cells.

We have previously shown that PML-RARa recruits HDAC3 to its target genes (31). The interaction with NuRD not only leads to chromatin remodeling and changes in DNA methylation but also causes a recruitment of two additional HDAC enzymes, which display differences in substrate specificity with respect to HDAC3 (18). This might expand the ability of PML-RARa to remove acetyl groups from histone tails, thus preparing them for further modifications. We have recently documented increased epigenetic marks (such as H3K27me) upon PML-RARa expression (32). The data presented here suggest that NuRD recruitment at target genes is required not only for PML-RARa-induced DNA methylation but also for deposition of Polycomb repressive marks. Interestingly, genetic analyses in Drosophila have demonstrated that the ATPase dMi-2 participates in Polycomb repression (20), thus anticipating a decade ago a role of NuRD in histone deacetylation and chromatin changes that in turn allow binding of Polycomb protein complexes. In leukemic cells, PML-RARa recruits both NuRD and Polycomb complexes to its target genes (32) and coordinates enzymatic activities to ensure a stable epigenetic gene silencing. Our kinetic analysis of corepressor occupancy suggests a precise sequence of events that occur at target genes, with NuRD-mediated DNA methylation or its stability. We suggest that PML-RARa represses gene transcription through several distinct mechanisms, including histone deacetylation, DNA methylation, histone modification, chromatin compaction, and heterochromatinization. The data presented here demonstrate that the NuRD complex plays a pivotal role in both the establishment and maintenance of aberrant epigenetic silencing imposed by PML-RARa and will help in identifying potential molecular targets of intervention in cancer.
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