Dual Functions of Histone-Lysine N-Methyltransferase Setdb1 Protein at Promyelocytic Leukemia-Nuclear Body (PML-NB)

MAINTAINING PML-NB STRUCTURE AND REGULATING THE EXPRESSION OF ITS ASSOCIATED GENES

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Background: Setdb1 regulates gene expression with various transcription factors.
Results: Setdb1 is a constitutive member of PML-NB and suppresses Id2 expression.
Conclusion: Setdb1 maintains PML-NB structure and concurrently controls PML-NB-associated genes.
Significance: This provides the mechanism of Setdb1 being involved in PML-mediated transcriptional regulation.

Setdb1/Eset is a histone H3 lysine 9 (H3K9)-specific methyltransferase that associates with various transcription factors to regulate gene expression via chromatin remodeling. Here, we report that Setdb1 associates with promyelocytic leukemia (Pml) protein from the early stage of mouse development and is a constitutive member of promyelocytic leukemia (PML)-nuclear bodies (PML-NBs) that have been linked to many cellular processes such as apoptosis, DNA damage responses, and transcriptional regulation. Arsenic treatment, which induces Pml expression, decreases Setdb1 degradation, causing Setdb1 signals to disappear. Setdb1 knockdown resulted in dismantlement of PML-NBs. Immunoprecipitation results demonstrated physical interactions between Setdb1 and Pml. Chromatin immunoprecipitation revealed that, within the frame of PML-NBs, Setdb1 binds the promoter of Id2 and suppresses its expression through installing H3K9 methylation. Our findings suggest that Setdb1 performs dual, but inseparable, functions at PML-NBs to maintain the structural integrity of PML-NBs and to control PML-NB-associated genes transcriptionally.

The methylation of lysine residues in histone proteins is a pivotal phenomenon in several cellular processes, including heterochromatin formation, X-chromosome inactivation, and transcription regulation (1). The specific pattern of lysine methylation can determine whether the local chromatin assumes a more compact or a more relaxed state. Of the various methylatable lysines, trimethylated lysine 9 of histone H3 (H3K9me3) is highly enriched in pericentric heterochromatin and is considered a marker of heterochromatin (2, 3). Correct formation of heterochromatin is necessary for maintaining chromosome stability and integrity and is crucial for the proper segregation of chromosomes during mitosis (4) and recombination events in fission yeast (5). Thus far, several histone-lysine methyltransferases (HKMTases)<sup>2</sup> specific for H3K9 residues have been identified, including Suv39h1 (6) and G9a (7).

Setdb1/Eset is an H3K9-specific HKMTase. Since its identification through its interaction with the ETS transcription factor ERG (8), various transcriptional regulators have been identified that are associated with Setdb1. Setdb1 interacts with the KAP1 corepressor that is attracted to target regions by KRAB zinc finger proteins, the largest family of sequence-specific DNA binding repressors (9), and contributes to HP1-mediated silencing of euchromatic genes (10). A recent study showed that KAP1 autosomally its bromodomain to recruit the NuRD complex and Setdb1, establishing a silent chromatin state at KAP1 target genes (11).

Interactions between Setdb1, Hdac1/2, and the mSin3A/B transcriptional corepressor result in a protein complex that participates in multiple transcriptional repression pathways (12). Sp3 also recruits Setdb1 to its target regions to regulate gene expression (13). In fact, most studies have shown that Setdb1 proteins are recruited to target genomic loci to induce heterochromatin formation. In addition to the local level function in regulating chromatin structure, as we recently showed (14), Setdb1 has been shown to localize to pericentric regions in germ line stem cells of Drosophila and regulate heterochromatin structure systemically. Setdb1 is also involved in the maintenance of heterochromatin structure during DNA replication through its associations with MBD1 and CAF-1 (15). These

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<sup>2</sup>The abbreviations used are: HKMTase, histone-lysine methyltransferase; PML-NB, promyelocytic leukemia-nuclear body; mESC, mouse embryonic stem cell; mEF, mouse embryonic fibroblast; HLH, helix-loop-helix; IP, immunoprecipitation; SUMO, small ubiquitin-like modifier.
observations implicate Setdb1 in diverse cellular processes. Mouse embryos that lack functional Setdb1 genes die around peri-implantation (around 4.5–5.5 days post-coitum) (16). Setdb1 dysfunction produces the earliest lethality relative to that of other H3K9-specific HKMTases, such as Suv39h1 (non-essential) (17) and G9a (mutant embryos die around day 9.5) (18), underscoring the developmental importance of Setdb1.

Promyelocytic leukemia (Pml) protein is a tumor suppressor that localizes to punctate nuclear structures called PML nuclear bodies (PML-NBs) that are interspersed in chromatin (for review see Refs. 19, 20). PML-NBs are proteinaceous structures found in most mammalian cell nuclei and typically number between 1 and 30 bodies per nucleus, depending on the cell type and cell cycle phase. The gene encoding the PML protein is situated at the breakpoint of a common genetic translocation found in patients with acute promyelocytic leukemia (21). Many different proteins associate with PML-NBs; consequently, PML-NBs have been implicated in various biological processes such as the induction of cellular senescence and apoptosis, the antiviral response, and the maintenance of genome stability (22). Many different transcription factors and regulators localize to PML-NBs (23). Recent studies suggest that PML-NBs specifically associate with the MHC class I gene cluster region (24) and regulate transcription by organizing the genes contained within that locus into distinct high order chromatin loop structures that are more permissive to transcription (25). In contrast, PML-NBs are also sites of localization for transcriptional corepressors and heterochromatin-bound proteins, such as HP1 (26). At a certain point in the cell cycle, PML-NBs are juxtaposed to pericentric heterochromatin regions and may function in the reestablishment of condensed heterochromatin (27). However, even in light of these observations, the molecular mechanism underlying PML-mediated transcriptional regulation remains largely unknown.

Here, we demonstrate that Setdb1 interacts with Pml proteins from the preimplantation stage in mice. Several lines of evidence support that Setdb1 is a stable member of PML-NBs as follows. First, Setdb1 knockout resulted in dismantlement of PML-NB structures. Second, arsenic treatment, which induces Pml degradation, caused Setdb1 signals to disappear. Third, immunoprecipitation (IP) experiments demonstrated a physical interaction between Setdb1 and Pml. We found that Setdb1 negatively regulated id2 gene expression; within the frame of PML-NB, Setdb1 associated with the id2 promoter and repressed its expression through imposing H3K9me3 onto the region. Our results demonstrated that the structural integrity of PML-NB was necessary for a proper regulation of id2 expression by the Setdb1 protein. These findings may serve as the cornerstone for understanding the diverse functions of PML-NBs and, more importantly, the molecular and biochemical roles of PML in acute promyelocytic leukemia pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Immunostaining**—The list of antibodies we used was as follows: anti-Setdb1 (07-378, Upstate; ab12317, Abcam), anti-Myc (A21208, Molecular Probes), α-tubulin (T-5168; Sigma), anti-PML (sc-5621, Santa Cruz Biotechnology; ab50637; Abcam), β-actin (sc-47778, Santa Cruz Biotechnology), and anti-H3K9me2 (ab1220, Abcam).

For immunostaining, cultured cells or mouse embryos were fixed in 4% (w/v) formaldehyde for 15 min at room temperature (RT) and washed in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBST, Fisher). After permeabilization for 30 min in PBS containing 1% Triton X-100 (MP Biomedicals), samples were blocked in 2% BSA/PBS (Sigma) for 1 h at RT and incubated for 1.5 h at 37 °C with primary antibodies. Alexa Fluor 488- or 594-conjugated secondary antibodies (1:300, Molecular Probes) were used. Stained samples were mounted on glass slides with mounting media containing DAPI (Vectasheild). Samples were observed with Carl Zeiss Axiosvert 200 M fluorescence microscope equipped with Apotome apparatus. Images were captured digitally and merged using Axiovision (version 4.7) or Adobe Photoshop software (version 7.0). Fluorescence intensity profile was measured using the profiling tool in Axiovision software.

**Mouse Embryo Collection, Microinjection, and in Vitro Culture**—Mouse oocytes and early embryos were collected from superovulated BCF1 (C57BL/6 × CBA/CA) females as described previously (28, 29). Briefly, female BCF1 mice at 5 weeks of age were injected with 5 IU of pregnant mare serum gonadotrophin, followed by 5 IU of human chorionic gonadotropins 48 h apart, and mated with male mice. Successful mating was determined the following morning by detection of a vaginal plug. Eighteen to 20 h after human chorionic gonadotropin injection, mouse zygotes were collected from mouse oviduct and transferred to M2 medium (Sigma) containing 0.1% (w/v) hyaluronidase to remove cumulus cells. Embryos were cultured into M16 medium (Sigma) at 37 °C, 5% CO2 in air and, if needed, collected according to standard procedures on optical stages. Unfertilized metaphase II stage oocytes were collected from ampullae of superovulated females without mating. Microinjection was performed 24 h after human chorionic gonadotropin injection (30). To visualize the pronuclei, cumulus-free zygotes were centrifuged at 13,000 rpm for 5 min. The concentration of microinjection DNA was adjusted to 5 ng/μl.

**RT-PCR and Knockdown Experiments**—Total RNAs were extracted from mouse embryos and cultured cells using RNeasy mini kit (Qiagen). Twenty embryos in each stage were used for RNA isolation. RT-PCR primers are listed in supplemental Table 1.

For introduction of knockdown constructs into cells, we used Lipofectamine and Plus reagent (Invitrogen) or Lipofectamine RNAiMax (Invitrogen). The list of knockdown constructs we used was as follows: Setdb1 (Open Biosystems and Dharmacon), Pml (Open Biosystems and Dharmacon), and GFP (Dharmacon). For immunofluorescent observations of knockdown effects, we cotransfected mEF cells with GFP expression vectors and the knockdown constructs at a 1:10 ratio in mass; hence, those of cells expressing GFP proteins were supposed to necessarily express knockdown constructs.

**Immunoprecipitation**—Cells were lysed using lysis buffer (0.5% Nonidet P-40, 50 mM Tris-Cl, 10% glycerol, 0.1 mM EDTA, 15 mM NaCl), and the whole cell lysates were collected. Ten μg of Setdb1 antibody (07-378, Upstate) or Pml antibody (sc-5621, Santa Cruz Biotechnology; ab50637, Abcam) was reacted with
100 μg of the lysates for 4 h at 4 °C. Fifty μl of protein A or protein A/G beads was added and incubated overnight at 4 °C. Beads were washed with the lysis buffer and subjected to Western blotting. As a control, rabbit and mouse IgGs were used.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP—NIH3T3 cells were harvested and fixed with 1% of formaldehyde for 10 min at RT. Fixed cell suspension was added to 0.125 M glycine and incubated for 5 min at RT. The cells were washed with PBS and were resuspended in ChIP lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate). Chromatin was sheared using a sonicator (Fisher) to carry 200–1,000-bp-long DNA fragments. Immunoprecipitation was conducted with 10 μg of normal rabbit IgG (Pierce), anti-Setdb1, and anti-PML (Santa Cruz Biotechnology) antibody with rotation overnight at 4 °C. Fifty μl of protein A/G-agarose beads were incubated with 20 μg of salmon sperm DNA for 10 h at 4 °C. Immunocomplexes were washed with ChIP lysis buffer, ChIP lysis high salt buffer (ChIP lysis buffer plus 500 mM NaCl), LiCl/detergent solution (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA), and TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) in succession. The complexes were incubated in 200 μl of TE containing 20 μg of RNase at 37 °C for 30 min and then in 500 μl of elution buffer (1% SDS, 0.1 M NaHCO₃). Twenty μl of 5 M NaCl was added to the eluent and incubated at 65 °C for at least 6 h to reverse cross-links. After precipitation, the eluent was dissolved in 178 μl of TE containing 20 μl of 10× proteinase K buffer (0.1 M Tris-HCl, pH 7.8, 50 mM EDTA, 5% SDS) and 2 μl of 20 μg/μl proteinase K at 50 °C for 30 min. Harvested DNAs were used as templates for PCR amplification. For primers used in PCR, see supplemental Table 2.

For sequential ChIP experiments (ReChIP), cross-linked chromatin fragments were incubated with primary antibody and protein A-agarose beads for at least 10 h at 4 °C on a rotating wheel. The immunoprecipitated complexes were eluted by incubation with 10 mM DTT at 37 °C for 30 min and diluted 1:20 in ChIP lysis buffer. Eluates were then re-immunoprecipitated with second antibody. Harvested DNA was used as templates for PCR.

RESULTS

Setdb1 Proteins Dynamically Redistribute during Mouse Preimplantation Stage—We examined in early mouse embryos the expression pattern of Setdb1 protein whose structure is shown in Fig. 1A. RT-PCR results showed that Setdb1 transcripts from maternal stock gradually decreased before zygotic transcription of Setdb1 was initiated at the blastocyst stage (Fig. 1B), which was in agreement with our previous results (16). Similarly, the level of Setdb1 protein, which was initially high, decreased during the same period (Fig. 1C). Suv39h1 and G9a, which also encode H3K9-specific HKMTases, are characterized by bursts of transcription by the blastocyte. However, when we used the same number (n = 40) of embryos used to detect Setdb1 protein, we could not detect a band for G9a or Suv39h1 in the Western blot analysis. In contrast, all three HKMTases were detected at comparable levels in cultured NIH3T3 cells (data not shown). These results suggest that among the redundant H3K9 HKMTases, Setdb1 prevails in early mouse embryos.

Immunostaining of mouse embryos showed that Setdb1 signals were more prominent in the nucleus (Fig. 1D, panels a–c). This pattern changed around the morula stage, when the diffuse nuclear signals declined (Fig. 1D, panel d), coinciding with the drain of the maternal stock of Setdb1 proteins (Fig. 1C). At the blastocyst stage, Setdb1 reappeared as a few punctate signals (Fig. 1D, panel e), which was completely different from the earlier diffuse pattern. The punctate signals were exclusively nuclear (Fig. 1D, panel f), and there was no regional preference for the inner cell mass cells or trophectoderm cells in the blastocyst.

Setdb1-Pml Association Begins as Early as the Cleavage Stages of Development—We sought to determine the nuclear structure to which Setdb1 proteins were localized. Of several candidates forming punctate signals in the nucleus, we took note of PML-NBs by the size and the number of the structure (19). The expression pattern of Pml proteins during the preimplantation stage of development has not been reported. RT-PCR results indicated that early mouse embryos had Pml transcripts, which progressively declined as embryos cleaved, reaching the lowest level by the blastocyst stage (Fig. 1E). Immunostaining showed that Pml signals were scattered in the nucleus at earlier cleavage stages (Fig. 1F). From around the 8-cell stage onward, Pml proteins flocked together to form a few speckles, the PML-NB structure, in the nucleus. The punctate PML-NB signals, similarly as the Setdb1 speckles (Fig. 1D, panel e), were equivalently distributed between inner cell mass cells and trophectoderm cells in the blastocyst. The diffuse-to-dot pattern shift of Pml signals was reminiscent of the dynamic change observed in the Setdb1 expression pattern during the same developmental window (Fig. 1D). Next, we examined whether Setdb1 and Pml proteins associated with each other during the preimplantation period. We microinjected Pml-myc expression plasmids into the pronucleus of mouse zygote. There are two Pml isoforms, isoform I and II, in mice (31, 32), and the isoform II cDNA was used to make the Pml-myc expression plasmids. In the resultant transgenic embryos, we were able to see Pml-myc signals colocalize with Setdb1 signals (Fig. 1G). The result indicates that Setdb1 interacts with Pml as early as the preimplantation stage of development.

The colocalization of Setdb1 and Pml proteins was also seen in cultured cells. mEFS were transiently transfected with Pml-myc plasmids and then subjected to a double immunostaining using α-Pml and α-Myc antibodies; the former was for detecting both endogenous and exogenous Pml proteins, and the latter was for the exogenous only. The two antibodies produced exactly the same fluorescent images in the case of Pml-myc-transfected cells (Fig. 2A, panels a–d). Therefore, we were convinced that the Pml-myc proteins behave equivalently to endogenous Pml proteins, so the Pml-myc proteins represent the endogenous Pml proteins properly in Pml-myc-expressing cells. When the Pml-myc-expressing cells were stained for Setdb1, as shown in Fig. 2A, panels e–h, Pml-myc signals were shown to be perfectly superimposed on endogenous Setdb1 signals in mEFS and other culture cells, including mESCs (supplemental Fig. S1). Colocalization of the proteins at PML-NBs was
confirmed with endogenous levels of Setdb1 and Pml (supplementary Fig. S2). The results demonstrate that the endogenous Pml and Setdb1 proteins are associated with each other.

The punctate signals of Setdb1 were observed to be present in every nuclei of mEFs, save some dividing cells with condensed chromosomes. When the cells were indexed by staining for dimethylated H3K9 (H3K9me2), which helps mark a boundary between the early prophase cells the late prophase cells (33, 34), all H3K9me2-positive cells contained punctate Setdb1 signals. Even the H3K9me2-negative cells with condensed chromosomes, as judged to be at late prophase of the cell cycle, were positive for Setdb1 signals (Fig. 2 B). A similar result was obtained when the cells were stained for Pml. However, Pml (35–38) or Setdb1 signals were greatly reduced or almost absent in the metaphase and anaphase cells that had undergone a nuclear membrane breakdown. Therefore, we concluded that Setdb1 proteins associate with PML-NBs during the cell cycle except at the metaphase and the ensuing stages of mitosis.

**Setdb1 Proteins Are Required for the Maintenance of PML-NB Structure**—Arsenic trioxide (As2O3) induces sumoylation-triggered degradation of Pml proteins and thus dismantlement of the PML-NB structure (39). When we treated mEFs with arsenic, not only Pml but Setdb1 signals disappeared from the nucleus (Fig. 2 C). Western blot analysis showed that, among the four different Pml variants (40), the largest one, about ~120 kDa in molecular mass, was particularly affected by the arsenic treatment (Fig. 2 D). Given only two spliced forms of Pml transcripts present in mice (32), the possibility cannot be excluded that the largest band is a sumoylated form of Pml protein. Meanwhile, it turned out that there was no recognizable change in the level of Setdb1 proteins in the arsenic-treated...
cells, notwithstanding the disappearance of the punctate Setdb1 signals; so it appeared that the arsenic treatment tore down PML-NBs, with the Setdb1 proteins loose and scattered from them. RT-PCR results showed that both Pml and Setdb1 genes were steadily transcribed in these cells (Fig. 2E). As arsenic was withdrawn from the culture medium, the foci of Setdb1 and Pml proteins reappeared (Fig. 2C, panels g and o). Western blotting verified the restoration of Pml proteins in the arsenic-withdrawn cells (Fig. 2D). These results demonstrate that the movement of Setdb1 is tightly linked with Pml proteins.

The result showed that the reduced level of Pml proteins caused the disappearance of PML-NBs and Setdb1 signals in the arsenic-treated cells. On the contrary, to study the role of Setdb1 proteins in PML-NB formation, we performed Setdb1 knockdown (Set-R) experiments in mEFs. Results from RT-PCR and Western blotting showed that Setdb1 expression was markedly reduced in Set-R cells (Fig. 3A). However, the expression level of Pml was not changed, indicating that the reduced level of Setdb1 does not have any influence on Pml expression. mEFs were transiently transfected with GFP expression vector alone or combined with Set-R plasmids, with the relative amounts of each fixed at a 1:10 molar ratio, ensuring that all GFP-positive cells necessarily expressed the knockdown constructs. When the GFP-positive Set-R cells were examined, the numbers of Setdb1 and Pml dots were found to be significantly reduced to about 30% of the level in GFP control cells (p < 0.001; Fig. 3, B and C, respectively). To see whether diminished levels of Setdb1 proteins could interfere with the refor-
Suppression of PML-NBs, we introduced the Set-R constructs to the arsenic-treated cells immediately after arsenic withdrawal. As shown in Fig. 3D, the suppression of Setdb1 expression hindered the cells from rebuilding PML-NBs in Set-R cells after arsenic withdrawal. After arsenic withdrawal (see Fig. 2), mEFs were transfected either with GFP expression plasmid alone (GFP) or in combination with Set-R constructs (GFP/Set-R) before culture in normal growth medium for another 48 h. Nuclear boundaries of GFP-positive cells are indicated by dotted circles. Asterisks over the bars in B–D denote significant differences between groups (p < 0.001). n in B–D, the number of cells counted. E and F, immunoprecipitation (IP). Protein extracts from mEFs and NIH3T3 cells were immunoprecipitated using H9251–Setdb1 (E) or H9251–Pml (F) antibody, and the precipitates were analyzed by Western blotting using indicated antibodies. Arrows in A, E, and F point at the 180-kDa full-length Setdb1 band. Five to 15% of cell lysates were used for input in IP. IP-IgG/IP-Setdb1/IP-Pml, IP with either control IgG, α-Setdb1, or α-Pml antibody, respectively; FT, flow-through.

**Suppression of Id2 Expression by Setdb1 Requires PML-NB Structure to Be Unperturbed**—The expressions of some genes were known to be regulated by Pml (41). We measured the expression levels of the Pml target genes such as Id2, p21, and Jpo-1 in Set-R cells and found Id2, which was previously shown negatively regulated by Pml, exhibiting a 4-fold increase in the mRNA level in Set-R cells (Fig. 4A); as for the other two genes, however, we observed no difference in expression level. Id2, inhibitor of DNA binding 2 (42), a transcriptional regulator containing a helix-loop-helix (HLH) domain but not a basic domain, inhibits the functions of basic HLH transcription factors in a dominant-negative manner by suppressing their heterodimerizing partners through the HLH domain. We next examined Id2 downstream genes such as M-CSF and collagen 1α1 genes in Set-R cells, which were previously shown to be positively regulated by Id2 (43, 44), and clearly found their expressions markedly augmented in Set-R cells (Fig. 4B). Meanwhile, when we examined cells overexpressing Setdb1, we observed no change in Id2 expression levels (supplemental Fig. S3). We also examined Pml knockdown (Pml-R) cells and found that the Id2 gene was up-regulated (Fig. 4C). Similarly, we found that Id2 was increasingly expressed in the arsenic-treated
We next inspected whether Setdb1 could directly bind the promoter of the Id2 gene. For this, we performed ChIP followed by PCR analysis of enrichment of Setdb1 and Pml on seven different positions (r1–r7) along the Id2 genomic region (Fig. 4E). ChIP data showed that especially the r3 and r4 regions, which positioned at proximal promoter, were enriched with Setdb1 and H3K9me3 (Fig. 4F); it hints that Setdb1 binds the promoter and subsequently methylated H3K9 residues of the corresponding region. In Set-R cells, the association of Setdb1 protein with the promoter was significantly diminished (p < 0.05); the decreased levels of Setdb1 and H3K9me3 at the Id2 promoter might account for the increased expression of the Id2 gene in Set-R cells. The same region was enriched when ChIP was performed using α-Pml antibody in Set-R cells, which suggests that Setdb1 and Pml proteins associate to the same promoter region. Other positions such as r2 and r6–7 distant from the proximal promoter showed no evidence for Setdb1 binding.

We repeated the ChIP experiment with Pml-R chromatin sample and obtained a similar result as in the experiment with the Set-R chromatin sample (Fig. 4G).

To confirm the ChIP results, we also examined the as-treated cell samples (see Fig. 2C). RT-PCR results showed that Id2 transcript level was markedly increased in as-treated cells and lowered to a control level in as-withdrawn cells (Fig. 4H). When the r3–r7 positions were examined for Setdb1 and Pml in Setdb1 and Pml ChIP samples, the extent of enrichment was lowered at r3 and r4 regions in as-treated cells but restored to a control level in as-withdrawn samples (Fig. 4I). The result suggests that binding of Setdb1 and Pml to the Id2 promoter is dependent on the integrity of the PML-NB structure.
The observation that either Set-R or Pml-R resulted in the simultaneous displacement of both Setdb1 and Pml proteins from the Id2 promoter (Fig. 4, F and G) suggests that both proteins are implicated in the regulation of Id2 expression. To verify the joint operation by Setdb1 and Pml to silence Id2 expression, we performed with the Set-R chromatin sample a successive ChIP experiments, a primary ChIP with α-Setdb1 antibody followed by a secondary IP (ReChIP) with α-Pml antibody (α-Setdb1→α-Pml) and vice versa (α-Pml→α-Setdb1). As shown in Fig. 4J, ChIP-ReChIP results showed that both Setdb1 and Pml proteins interacted with or were in close contact to the Id2 promoter. The antibody binding was specific because ChIP-ReChIP experiments with Set-R chromatin sample did not immunoprecipitate Setdb1 or Pml fractions. Taken together, our findings strongly suggest that the unimpairedness of PML-NB structure is essential to properly repress the Id2 gene when down-regulated. Given the structural integrity of PML-NBs was almost intact, indicating that the integrity of PML-NBs was maintained back to normal (Fig. 2G). Based on these observations, it is of no doubt that Setdb1 is a steady constituent, not a fleeting component, of PML-NBs. Several lines of evidence solidify this notion. First, all PML-NB foci contained Setdb1 proteins with a spot density equivalent to that of Pml (Fig. 2A). Second, Setdb1 knockdown resulted in diminishment of PML-NBs in number (Fig. 3C). Third, the arsenic treatment, which led to the dismantling of PML-NBs, caused the punctate Setdb1 signals to vanish (Fig. 2C). Finally, Setdb1 and Pml signals colocalized throughout most of the cell cycle, even until the late prophase of mitosis (Fig. 2B). Based on these observations, it is of no doubt that Setdb1 is on very close terms with Pml and is indispensable to the PML-NB structure.

In addition, the importance of Setdb1 in the maintenance of the structural integrity of PML-NBs can be further emphasized by the following observations. Pml knock-out caused aberrant localization of other known associates of PML-NBs such as Sp100, CBP, BLM, and Daxx (47, 48), but the reverse was not true; in the cells that lacked other PML-NB components such as Sp100 and BLM, the punctate PML-NB signals remained almost intact, indicating that the integrity of PML-NBs was unimpaired in these cells (47, 49). To our knowledge, Setdb1 is the only protein save Pml itself that significantly affects the PML-NB structure when down-regulated. Given the structural role of Setdb1 in the formation of PML-NBs, and considering its own fundamental function in H3K9 methylation and the
following assembly of local heterochromatin, we hypothesize that Setdb1 proteins serve as a molecular bridge coupling PML-NBs to chromatin; Setdb1 proteins position themselves in certain regions of chromatin to hold PML-NBs tightly onto certain nuclear domains (see below).

Setdb1 has been shown to bind sumoylated KAP1 (11) and SP3 (13) via its SUMO-interactive motif. Thus, it appears that Setdb1 is, in SUMO-conjugated forms, able to associate with its partner proteins and also bind other sumoylated partners via the SUMO-interactive motif-mediated docking mechanism. Interestingly, Setdb1 exhibited increased methylating activity toward histone H3 in the presence of sumoylated KAP1 (11). If this is the case, Setdb1 proteins that bind SUMO-conjugated transcription factors in PML-NB could more efficiently methylate surrounding nucleosomes. HP1 proteins then bind specifically to these methylated H3K9 marks (see Fig. 5), and local HP1 recruitment is sufficient to mediate heterochromatin formation (50). It has been reported that PML-NBs colocalize with HP1 proteins (26, 27). Therefore, it suggests that not only does Setdb1 negatively regulate gene expression by repressing chromatin structure, but it plays a stabilizing role as riveting such colossal PML-NBs to several adjacent spots of chromatin. To date, numerous proteins with diverse functions have been found to localize to PML-NBs (51); however, to our knowledge, Setdb1 is the only H3K9-specific HKMTase found. Given its function as an H3K9-specific HKMTase, we assume that Setdb1 proteins anchor themselves, with the aid of recruited HP1 proteins, to certain regions of chromatin on which the gigantic PML-NBs are stabilized. Recently, Setdb1 conjugation by SUMO has also been observed in its interaction with Oct4 (45).

Our results suggest that the structural integrity of PML-NB is a prerequisite for proper regulation of Id2 expression by Setdb1. Set-R or Pml-R alone was sufficient to disrupt the PML-NB structure (Fig. 3C and supplemental Fig. S4), and the resulting dysfunction of PML-NBs was linked to misregulation of Id2 gene expression. The protein levels of Setdb1 (Fig. 4C) and Pml (Fig. 3A) were not altered in Pml-R and Set-R cells, respectively, so these proteins are thought to exist unleashed from PML-NBs in the nucleus. Our result showed, however, that in their free form, without being involved in PML-NB, Setdb1 and Pml proteins are unable to take a seat on the Id2 promoter (Fig. 4, F, G, and I). It is less likely that Setdb1 and Pml proteins themselves could autonomously scan the genome to choose specific loci on which to act. Instead, as illustrated in Fig. 5, there are possibly certain proteins such as transcription factors that could bind regulatory DNA regions with sequence specificity and at the same time as Setdb1 protein. Upon stimulation, this regulatory protein may first bind to the promoter of a target gene. Then, the DNA–protein complex is likely to be drawn in to a nearby PML-NB because of the higher Setdb1 activity enriched in PML-NB. Considering the gigantic size of PML-NBs, it is unlikely for PML-NB to move in person to find the DNA-protein complex. The association of Setdb1 with the regulatory protein transforms the genomic region into a repressive state by H3K9me3 installment and subsequent recruitment of HP1 proteins, ultimately leading to turn off of the target gene expression. At the repressive condition like this, the regulatory proteins may no longer stay at the region and thus drift away.

It is tempting to conceive that, in addition to the role of an architectural platform on which PML-NB is established and stabilized, Setdb1 proteins could serve as a core participant of PML-NB to regulate the expression of Pml-associated genes, for the most part negatively through placing repressive H3K9me3 marks. There are a number of genes known to be negatively regulated by Pml (41), and there is a possibility that most of the Pml-regulated genes belong to a common negative control mechanism and that PML-NB adopts, as the common mechanism, the strategy of Setdb1-mediated repression of gene expression. In support of this notion, we found that Setdb1 negatively controlled the expression of Id2 through binding to the promoter and labeling the region with H3K9me3 and that functional PML-NB is needed for Setdb1 to act on the Id2 promoter (Fig. 4). If this Setdb1-mediated repression mechanism is truly general for other Pml-controlled genes within the frame of PML-NBs, the mechanism could be considered to be very efficient from the epigenetic point of view in that a single regulatory mechanism is uniformly applicable to a number of genes as a package that locate at different genomic loci.

REFERENCES

1. Martin, C., and Zhang, Y. (2005) Nat. Rev. Mol. Cell Biol. 6, 838–849
2. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001) Nature 410, 120–124
3. Peters, A. H., Kubicek, S., Mechtler, K., O’Sullivan, R. J., Derijck, A. A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J. H., and Jenuwein, T. (2003) Mol. Cell 12, 1577–1589
4. Pidoux, A. L., and Allshire, R. C. (2005) Philos. Trans. R. Soc. Lond. B Biol. Sci. 360, 569–579
5. Iia, S., Yamada, T., and Grewal, S. I. (2004) Cell 119, 469–480
6. Aagaard, L., Laible, G., Selenko, P., Schmid, M., Dorn, R., Schotta, G., Kuhfittig, S., Wolf, A., Lebersorger, A., Singh, P. B., Reuter, G., and Jenuwein, T. (1999) EMBO J. 18, 1923–1938
7. Tachibana, M., Sugimoto, K., Fukushima, T., and Shinkai, Y. (2001) J. Biol. Chem. 276, 25309–25317
8. Yang, L., Xia, L., Wu, D. Y., Wang, H., Chansky, H. A., Schubach, W. H., Hicklestein, D. D., and Zhang, Y. (2002) Oncogene 21, 148–152
9. Urrutia, R. (2003) Genome Biol. 4, 231
10. Schultz, D. C., Ayyanathan, K., Negorev, D., Maul, G. G., and Rauscher, F. J., 3rd. (2002) Genes Dev. 16, 919–932
11. Ivanov, A. V., Peng, H., Yurchenko, V., Yap, K. L., Negorev, D. G., Schultz, D. C., Paulkowski, E., Fredericks, W. J., White, D. E., Maul, G. G., Sadowsky, M. J., Zhou, M. M., and Rauscher, F. J., 3rd. (2007) Mol. Cell. 28, 823–837
12. Yang, L., Mei, Q., Zielinska-Kwiatkowska, A., Matsui, Y., Blackburn, M. L., Benedetti, D., Krumm, A. A., Taborsky, G. J. Jr., and Chansky, H. A. (2003) Biochem. J. 369, 651–657
13. Stodieck, B., Sapetschnig, A., Wink, C., Krüger, L., and Suske, G. (2008) EMBO Rep. 9, 899–906
14. Yoon, J., Lee, K. S., Park, J. S., Yu, K., Paik, S. G., and Kang, Y. K. (2008) PLoS ONE 3, e2234
15. Sarraf, S. A., and Stancheva, I. (2004) Mol. Cell. 15, 595–605
16. Dodge, J. E., Kang, Y. K., Beppu, H., Lei, H., and Li, E. (2004) Mol. Cell. 24, 2478–2486
17. Peters, A. H., O’Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schöfer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. (2001) Cell 107, 323–337
18. Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., Fukuda, M., Takeda, N., Niida, H., Kato, H., and Shinkai, Y. (2002) Genes Dev. 16, 1779–1791
19. Bernardi, R., and Pandolfi, P. P. (2007) Nat. Rev. Mol. Cell Biol. 8, 1006–1016
Setdb1 Function in PML-NB

20. Ma, D. K., Chiang, C. H., Ponnuasamy, K., Ming, G. L., and Song, H. (2008) Stem Cells 26, 2131–2141.
21. de Thé, H., Chomienne, C., Lanotte, M., Degos, L., and Dejean, A. (1990) Nature 347, 558–561.
22. Dellaire, G., and Bazett-Jones, D. P. (2004) BioEssays 26, 963–977.
23. Zhong, S., Salomoni, P., and Pandolfi, P. P. (2000) Nat. Cell Biol. 2, E85–E90.
24. Shiel, C., Islam, S. A., Vatcheva, R., Sasieni, P., Sternberg, M. J., Freemont, P. S., and Sheer, D. (2001) J. Cell Sci. 114, 3705–3716.
25. Kumar, P. P., Bischof, O., Purbey, P. K., Notani, D., Urlaub, H., Dejean, A., and Galande, S. (2007) Nat. Cell Biol. 9, 45–56.
26. Seeler, J. S., Marchio, A., Sitterlin, D., Transy, C., and Dejean, A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 7316–7321.
27. Luciani, J. J., Depetris, D., Usson, Y., Metzler-Guillemain, C., Mignon-Ravix, C., Mitchell, M. J., Megarbane, A., Sarda, P., Sirma, H., Moncla, A., Feunteun, J., and Mattei, M. G. (2006) J. Cell Sci. 119, 2518–2531.
28. Jeong, Y. S., Yeo, S., Park, J. S., Lee, K. K., and Kang, Y. K. (2007) Dev. Dyn. 236, 1509–1516.
29. Yeo, S., Lee, K. K., Han, Y. M., and Kang, Y. K. (2005) Mol. Cells 20, 423–428.
30. Kang, Y. K., Park, J. S., Lee, C. S., Yeom, Y. I., Chung, A. S., and Lee, K. K. (1999) J. Biol. Chem. 274, 36585–36591.
31. Ebrahimian, M., Mojtabahzadeh, M., Bazett-Jones, D., and Dehghani, H. (2010) Cells Tissues Organs 192, 374–381.
32. Goddard, A. D., Yuan, J. Q., Fairbairn, L., Dexter, M., Borrow, J., Kozak, C., and Solomon, E. (1995) Mamm. Genome 6, 732–737.
33. Duan, Q., Chen, H., Costa, M., and Dai, W. (2008) J. Biol. Chem. 283, 33585–33590.
34. Jeong, Y. S., Cho, S., Park, J. S., Ko, Y., and Kang, W. Y. (2010) Genes Cells 15, 181–192.
35. Eskiw, C. H., Dellaire, G., and Bazett-Jones, D. P. (2004) J. Biol. Chem. 279, 9577–9585.
36. Eskiw, C. H., Dellaire, G., Mymryk, J. S., and Bazett-Jones, D. P. (2003) J. Cell Sci. 116, 4455–4466.
37. Everett, R. D., Earnshaw, W. C., Pluta, A. F., Sternsdorf, T., Ainsztein, A. M., Carmena, M., Ruchaud, S., Hsu, W. L., and Orr, A. (1999) J. Cell Sci. 112, 3443–3454.
38. Everett, R. D., Lomonte, P., Sternsdorf, T., van Driel, R., and Orr, A. (1999) J. Cell Sci. 112, 4581–4588.
39. Chen, G. Q., Shi, X. G., Tang, W., Xiong, S. M., Zhu, J., Cai, X., Han, Z. G., Ni, J. H., Shi, G. Y., Jia, P. M., Liu, M. M., He, K. L., Niu, C., Ma, J., Zhang, P., Zhang, T. D., Paul, P., Naoe, T., Kitamura, K., Miller, W., Waxman, S., Wang, Z. Y., de The, H., Chen, S. J., and Chen, Z. (1997) Blood 89, 3345–3353.
40. Ito, K., Bernardi, R., Morotti, A., Matsuoka, S., Saglio, G., Ikeda, Y., Rosenblatt, J., Avigan, D. E., Teruya-Feldstein, J., and Pandolfi, P. P. (2008) Nature 453, 1072–1078.
41. Cairo, S., De Falco, F., Pizzo, M., Salomoni, P., Pandolfi, P. P., and Meroni, G. (2005) Oncogene 24, 2195–2203.
42. Ruzinova, M. B., and Benezra, R. (2003) Trends Cell Biol. 13, 410–418.
43. Gratze, P., Dechend, R., Stocker, C., Park, J. K., Feldt, S., Shagdarsuren, E., Wellner, M., Gueler, F., Rong, S., Gross, V., Obst, M., Pheehm, R., Alenina, N., Zenclussen, A., Titze, I., Small, K., Yokota, Y., Zenke, M., Luft, F. C., and Muller, D. N. (2008) Circulation 117, 2645–2656.
44. Kurowska-Stolarska, M., Distler, J. H., Jüngel, A., Rudnicky, W., Neumann, E., Pap, T., Wenger, R. H., Michel, B. A., Müller-Ladner, U., Gay, R. E., Raslinski, W., Gay, S., and Distler, O. (2009) Arthritis Rheum. 60, 3663–3675.
45. Yeap, L. S., Hayashi, K., and Surani, M. A. (2009) Epigenetics Chromatin 2, 12.
46. Melnick, A., and Licht, J. D. (1999) Blood 93, 3167–3215.
47. Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., 3rd, and Maul, G. G. (1999) J. Cell Biol. 147, 221–234.
48. Zhong, S., Müller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000) Blood 95, 2748–2752.
49. Zhong, S., Hu, P., Ye, T. Z., Stan, R., Ellis, N. A., and Pandolfi, P. P. (1999) Oncogene 18, 7941–7947.
50. Li, Y., Danzer, J. R., Alvarez, P., Belmont, A. S., and Wallrath, L. L. (2003) Development 130, 1817–1824.
51. Hofmann, T. G., and Will, H. (2003) Cell Death Differ. 10, 1290–1299.