Overlapping Roles of the Methylated DNA-binding Protein MBD1 and Polycomb Group Proteins in Transcriptional Repression of HOXA Genes and Heterochromatin Foci Formation

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Methylated DNA binding domain (MBD) proteins and Polycomb group (PcG) proteins maintain epigenetic silencing of transcriptional activity. We report that the DNA methylation-mediated repressor MBD1 interacts with Ring1b and hPc2, the major components of Polycomb repressive complex 1. The cysteine-rich CXXC domains of MBD1 bound to Ring1b and the chromodomain of hPc2. Chromatin immunoprecipitation analysis revealed that MBD1 and hPc2 were present in silenced Homeobox A (HOXA) genes which could be reactivated by knockdown of either MBD1 or hPc2, suggesting that MBD1 and hPc2 cooperate for transcriptional repression of HOXA genes.

In the nuclei of HeLa cells, MBD1 existed in close association with these PcG proteins in some heterochromatin foci, whereas an MBD1 mutant lacking the CXXC domains or an hPc2 mutant lacking the chromodomain lost this colocalization in foci. Use of the DNA demethylating agent 5-aza-2′-deoxycytidine abolished the formation of MBD1 foci but not PcG foci. Knockdown of MBD1 by small interfering RNAs did not affect the foci containing hPc2 and Ring1b, whereas the MBD1 foci were not influenced by knockdown of hPc2. These indicate that the heterochromatin foci showing MBD1 and hPc2 colocalization arise through the interaction of MBD1 and hPc2 and that the foci of MBD1 are separable from those of the PcG proteins per se. Our present findings suggest that MBD1 and PcG proteins have overlapping roles in epigenetic gene silencing and heterochromatin foci formation through their interactions.

Patterns of gene expression are stably inherited during somatic cell division through maintenance of the epigenetic state of the mammalian genome (1, 2). The two major epigenetic silencing pathways play essential roles in this process as mechanisms of cellular memory. One pathway involves DNA methylation and subsequent recognition by methylated DNA binding domain (MBD) proteins (3–5), whereas the other pathway is a repressive mechanism involving Polycomb group (PcG) protein complexes (6, 7).

Cytosine methylation in 5′-CpG-3′ dinucleotides is well correlated with gene repression and the formation of transcriptionally inactive chromatin (8). DNA methyltransferases (DNMTs) methylate genomic DNAs followed by binding of MBD family proteins (9, 10). To date, five members of the MBD family have been identified in mammals by the presence of highly conserved MBD sequences (3–5). Among these, MBD1 is known to act as a transcriptional repressor through cooperation of the MBD, cysteine-rich CXXC domains (CXXC1, CXXC2, and CXXC3), and a transcriptional repression domain (TRD) (11–13). The MBD of MBD1 binds a symmetrically methylated CpG sequence (14) and is also associated with a histone methyltransferase, Su(var)3-9h1, which methylates lysine 9 of histone H3 (H3K9) and heterochromatin protein 1 (15). Furthermore, the TRD of MBD1 produces a strong repressive activity by recruiting MBD1-containing chromatin-associated factor 1 (MCAF1)/ATFa-associated modulator (AM) (16, 17). MCAF1/AM complexes with another histone methyltransferase, SETDB1, and is required for trimethylation of H3K9 by this enzyme (18). In addition, there are at least five isoforms of MBD1 that are alternatively spliced in the region containing the three CXXC sequences (11). The CXXC domain was originally found in DNMT1 and the Trithorax group protein ALL-1 (also known as MLL) (11). Although the CXXC3 domain of MBD1 was shown to bind DNA (13, 19), the significance of the CXXC domains in MBD1 remains to be determined.

The Polycomb group (PcG) proteins were initially identified as regulators of the homeobox (HOX) genes during development.

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** The abbreviations used are: MBD, methylated DNA binding domain; PcG, Polycomb group; TRD, transcriptional repression domain; H3K9, lysine 9 of histone H3; PRC, Polycomb repressive complexes; RT, reverse transcription; NLS, nuclear localization signal; DNMT, DNA methyltransferase; siRNA, small interfering RNA; GST, glutathione S-transferase; GFP, green fluorescent protein.
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in Drosophila melanogaster (20). These proteins are evolutionarily conserved in mammals and are expected to maintain the heritable repressive state of the HOX genes and various growth-controlling genes (21, 22). Similarly to the DNA methylation system, PcG proteins are involved in a variety of phenomena, such as X-chromosome inactivation, genomic imprinting, and control of cell proliferation and differentiation (23–25). Biochemical and genetic studies have indicated that PcG proteins can be classified into at least two Polycomb repressive complexes (PRC) named PRC1 and PRC2 (25–27). At the initiation of gene silencing, PRC2, which contains EED, EZH2, YY1, and SU(Z)12, is recruited to methylate lysine 27 of histone H3 (H3K27) via the histone methyltransferase EZH2 present in the complex. At the maintenance stage, PRC1, which contains Ring1b, hPc2, and BMI1, binds the trimethylated H3K27 through recognition by hpc2 (28, 29). The sequential actions of these PRCs result in stable maintenance of gene silencing. It is of interest that EZH2 and BMI1 are overexpressed in certain cancers and have been implicated in the process of tumorigenesis (30), suggesting that PcG proteins are crucial for cell regulation.

Despite the biological importance of MBD proteins and PcG proteins, the functional relationship of these repressive proteins has remained unknown. A recent study showed that EZH2 in PRC2 directly interacts with DNMTs and maintains CpG methylation at EZH2-targeted promoters (31), suggesting that EZH2 directly controls DNA methylation of EZH2 target genes. Mice embryos deficient for Eed changed the DNA methylation status of specific CpGs in differentially methylated regions at imprinted loci (32). As well, the association of BMI1 with PcG bodies, sites of the PcG complex accumulation mostly located in heterochromatin regions, requires DNMT1 as well as EZH2 and EED (33). This suggests that DNMT1 is involved in the formation of silent chromatin by PcG proteins. Collectively, these observations suggest that the two epigenetic silencing pathways are mechanistically linked. During an investigation of the roles of the CXXC domains of MBD1, we found that MBD1 interacts with Ring1b and hpc2, which are key molecules in PRC1. Here, we present evidence demonstrating the importance of the overlapping roles of MBD1 and PcG proteins in transcriptional repression of HOXA genes and heterochromatin foci formation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Yeast strain AH109 carrying pAS2–1-CXXC domain of MBD1 (amino acids 150–268 in isoform v1) was transformed with the mouse E17 whole embryonic cDNA libraries constructed in pACT2 (Clontech). Plasmids harboring cDNA were recovered from both histidine- and adenine-positive colonies and were used for DNA sequencing analysis.

Plasmids—The cDNA for hPc2 and Ring1b were cloned into pcDNA3 (pcDNA3-FLAG-hPc2 and pcDNA3-DsRed- monomer-Ring1b). The chromodomain of hPc2 (amino acids 3–71) was subcloned into pCMV-GAL4. pSilencer3.1 puro vector (Ambion) was used to express small hairpin RNAs. Target sequences are indicated in supplemental Table 1. The pCGN-MBD1, pEGFP-MBD1, and pCMV-GAL4-CXXC of MBD1 were previously described (13).

Cell Culture—HeLa cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s minimum essential medium and Ham’s F-12 nutrient medium (Sigma) supplied with 10% (v/v) heat-inactivated fetal bovine serum.

Transfection and Cell Treatment—HeLa cells were transfected with plasmid DNAs by using a liposome-mediated gene transfer method. For the luciferase assay, HeLa cells (1.0 × 10⁵ cells) were transfected with siRNA expression vector (2.0 µg) with FuGene6 (Roche Applied Science) in a 6-well plate, and after 24 h the cells were diluted to 1:2 and transferred to a 12-well plate under selection with puromycin (1.0 µg/ml). In addition, HeLa cells were treated with 5 µM 5-aza-2-deoxycytidine. After 3 days, the treated cells were used for immunofluorescent assay and reverse transcription (RT)-PCR.

Protein Expression—The cDNAs for Ring1b, hPc2, and the deletion mutants of hpc2 were cloned into pET28a (Novagen). The expression of these proteins, GST-fused MBD1v1 and GST-fused portions of MBD1, were performed as described previously (17).

Antibodies—The polyclonal antibodies against hpc2 were generated by immunizing a rabbit against His-tagged hpc2 (amino acids 59–466). For affinity purification of the antibodies, His-tagged hpc2 (amino acids 59–466) was coupled to HiTrap Protein G HP (GE Healthcare). Rabbit anti-MBD1 polyclonal antibodies were previously described (13). Mouse anti-Ring1b monoclonal antibodies were provided from Dr. H. Koseki. Other antibodies utilized were anti-MBD1 (Santa Cruz), anti-trimethylated H3K9, anti-trimethylated H3K27 (Upstate), anti-FLAG (M5) (Sigma), anti-His tag (Qiagen), anti-GST (DAKO), anti-amin A/C (Santa Cruz), anti-GAL4 (Santa Cruz), and anti-β-tubulin (Amersham Biosciences).

Immunoprecipitation—HeLa cells were treated with dimethyl 3,3’-dithiobispropionimidate-2HCl (5 mM) (Pierce) in phosphate-buffered saline, rinsed with an ice-cold buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl), and lysed with a hypotonic buffer (10 mM Tris-HCl (pH 7.9), 10 mM NaCl, 1.5 mM MgCl₂) supplemented with protease inhibitors for 10 min at 4 °C. The nuclei were collected by centrifugation (1500 rpm) at 4 °C for 10 min, mixed with buffer containing 20 mM Tris-HCl (pH 7.9), 420 mM NaCl, 5% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, and protease inhibitors, and then incubated with rotation for 30 min at 4 °C. After centrifugation (1500 rpm), the nuclei extracts were lysed using sonication with a radioimmuneprecipitation assay buffer (1% Nonidet P-40, 0.1% SDS, 500 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5% glycerol, 1% sodium deoxycholate, and protease inhibitors). The lysates were incubated for 1 h at 4 °C with specific antibodies or control IgG and then incubated for 1 h after the addition of 20 µl of protein A/G-agarose beads (Amersham Biosciences). After the washings, the bound proteins were detected by Western blot analysis.

In Vitro Binding and GST Pulldown Assay—Bacterially expressed GST and GST fusion proteins (1 µg) were immobilized on glutathione-agarose beads and incubated with His-tagged PcG proteins (1 µg) in a buffer containing 0.05% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 5% glycerol, 10
μM ZnCl₂, 1 mM dithiothreitol, and protease inhibitors for 1 h at 4 °C. The input indicates 10% of the His-tagged proteins.

**Immunofluorescent Analysis**—After being washed 2 times with phosphate-buffered saline (PBS), HeLa cells were similarly fixed 4% paraformaldehyde in PBS for 15 min at room temperature and then treated with 0.2% Triton X-100 for 5 min at 4 °C. After washing the cells with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin, we incubated them with specific antibodies in PBS containing 0.2% bovine serum albumin for 1 h at room temperature. Samples were analyzed with an Olympus IX71 microscope using LuminaVision software.

** Luciferase Assay**—At 48 h after transfection with a luciferase reporter plasmid and together with pCMV-GAL4 (CXXC domains of MBD1 or chromodomain of hPc2) and pcDNA3-FLAG-hPc2, HeLa cells were lysed in a buffer provided by the manufacturer (Promega). pRL-SV40, insertless pcDNA3, and pCMV-GAL4 were used as controls. Values are the means and S.D. of results from three independent experiments.

**Chromatin Immunoprecipitation**—HeLa cells (1 × 10⁶) were treated with dimethyl 3,3’-dithiobispropionimidate-2HCl (5 mM), rinsed, and then cross-linked by the addition of 1% formaldehyde at 37 °C for 10 min. Crude cell lysates were sonicated to generate 200–1000 bp of DNA fragments. Chromatin immunoprecipitation was performed with anti-MBD1, anti-hPc2 antibodies, or control IgG according to the manufacturer’s protocols (Upstate Biotechnology, Inc.). PCR amplification of human HOXA gene promoters was carried out for 35 cycles under the conditions of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C using specific set of primers described in supplemental Table 1. DNAs in input lysates were used as a positive control (input control).

**siRNA Knockdown of MBD1 and hPc2**—Twenty-one-nucleotide siRNA duplexes were designed to target mRNAs encoding human MBD1 and hPc2. The selected siRNA target sequences were submitted to human genome and EST databases to ensure the target specificities. These sequences were submitted to human genome and EST databases. Each experiment was carried out at least three times. The primer sets are listed in supplemental Table 1. The siRNAs for lamin A/C were previously reported (17). The siRNAs were transfected into the cells using Oligofectamine (Invitrogen) for 48 h.

**RT-PCR and Quantitative Real-time RT-PCR**—Total RNAs were isolated using Isogen (Nippon Gene). For cDNA synthesis, 5 μg of total RNAs was reverse-transcribed with Superscript III (Invitrogen) using oligo-dT or random oligo primers. The RT-PCR was carried out for 30 cycles. Quantitative real-time PCR of the target cDNAs was performed by the SYBR Green method using Power SYBR Green PCR Master Mix (Applied Biosystems). Each experiment was carried out at least three times. The -fold relative enrichment was quantified together with normalization by the β-actin level. The primer sets are listed in supplemental Table 2.

**Bisulfite Genomic Sequencing**—Genomic DNAs were treated with sodium bisulfite followed by PCR amplification with specific primers using AmpliTaq Gold (Applied Biosystems). The primers used were described in supplemental Table 2. The resulting PCR products were purified and directly sequenced.

**RESULTS**

**MBD1 Interacts with PcG Proteins**—To identify factors that interact with MBD1, we performed yeast two-hybrid screening using the region containing the CXXC domains (amino acids 150–268) as bait (Fig. 1A). From a screening of ~7 × 10⁶ independent transformants of 17-day-old mouse embryo cDNA libraries, we isolated a cDNA clone encoding Ring1b (amino acids 250–337). To confirm an interaction between MBD1 and Ring1b, we prepared His-tagged full-length Ring1b and subjected it to an in vitro pulldown analysis (Fig. 1B). Briefly, GST and GST-fused portions of MBD1 were immobilized on glutathione-agarose beads and incubated with His-Ring1b. Ring1b predominantly bound the CXXC1 and CXXC2 domains of MBD1, but not its MBD, CXXC3, and TRD domains, in agreement with the yeast two-hybrid screen.

Our previous alignment analysis revealed that the amino acid sequence of the CXXC3 domain of MBD1 shows high identity with that of the CXXC domain of ALL1/MLL (11). ALL1/MLL is one of the Trithorax group proteins and methylates histone H3 at lysine 4 (34, 35). It was previously reported that the region containing the CXXC domain of ALL1/MLL interacts with hPc2 (36). To test whether the CXXC domains of MBD1 can interact with hPc2, His-tagged hPc2 was prepared for an in vitro pulldown analysis (Fig. 1B). His-hPc2 specifically bound the CXXC3 domain as well as full-length MBD1. To further determine the region responsible for binding MBD1, we used two deletion mutants of hPc2, hPc2ΔN, and hPc2ΔC, which lacked the amino- and carboxyl-terminal regions of the protein, respectively (Fig. 1A). hPc2ΔC bound GST-MBD1, whereas hPc2ΔN lacking the chromodomain did not (Fig. 1C). These data suggest that the CXXC domains of MBD1 directly interact with Ring1b and hPc2, which are essential components of PRC1.

**MBD1 Forms Complexes with PcG Proteins in Vivo**—To confirm the interactions between MBD1 and PcG proteins in vivo, we performed an immunoprecipitation analysis. Hemagglutinin-tagged MBD1 and FLAG-tagged hPc2 were coexpressed in HeLa cells and immunoprecipitated by anti-MBD1 and anti-hPc2 antibodies, respectively. Western blot analysis revealed that MBD1 was present in the immunoprecipitates with hPc2 but not in control immunoprecipitates (Fig. 1D). hPc2 was detected in the immunoprecipitates with MBD1. Likewise, an immunoprecipitation analysis after coexpression of hemagglutinin-MBD1 and DsRed-tagged Ring1b revealed that MBD1 formed complexes with Ring1b (data not shown). To confirm whether exogenously expressed hPc2 and Ring1b form complexes in cells, immunoprecipitation of FLAG-hPc2 and DsRed-Ring1b was carried out (Fig. 1E). These PcG proteins were found to be mutually coprecipitated. We further investigated the association of endogenous MBD1 and hPc2 in HeLa cells without any overexpression. The coprecipitated bands for endogenous proteins were relatively faint and constant in repeated experiments (data not shown), probably due to the low biochemical solubility of endogenous MBD1 (16, 37). Together with the colocalization data (see Figs. 4 and 5), these results show that MBD1 and PcG proteins form complexes in vivo.
Cooperative Interaction of MBD1 and hPc2 for a Transcriptional Role—We tested whether combinations of the CXXC domains of MBD1 and hPc2 and of the chromodomain of hPc2 and MBD1 functionally interact for transcriptional control using luciferase reporter experiments (Fig. 2A). We expressed GAL4-fused CXXC domains of MBD1 (GAL4-CXXC) or the GAL4-fused chromodomain of hPc2 (GAL4-chromo) in HeLa cells. Western blot analyses revealed that GAL4-CXXC and GAL4-chromo were appropriately expressed in this assay (Fig. 2B–D). The effects of the combinations were examined by using a *Pho tinus pyralis* luciferase reporter containing five GAL4 binding elements upstream of the human p16 gene promoter. The p16 gene is targeted by CpG hypermethylation and subsequent binding of MBD1 or by PcG proteins in certain cancer cells (10, 30, 38, 39). GAL4-CXXC alone tended to decrease the luciferase activity in a dose-dependent manner (Fig. 2B). To assess the functional implication of the MBD1-hPc2 association, we checked the effect of exogenous hPc2 on the CXXC-based transcription. Coexpression of FLAG-hPc2 moderately decreased the CXXC-based luciferase activities, consistent with the interaction of MBD1 with hPc2. To elucidate the cooperative role of MBD1 and hPc2, we performed selective knockdown of endogenous hPc2 or MBD1 using pSilencer3.1puro vectors producing specific short hairpin RNAs (Fig. 2C). Western blot analyses revealed the effectiveness of the MBD1 or hPc2 knockdown (supplemental Fig. 1A). Knockdown of hPc2 constantly increased the CXXC-based transcription compared with the level with CXXC alone.

To further examine the transcriptional effect of the chromodomain of hPc2, we expressed GAL4-chromo in HeLa cells (Fig. 2D). GAL4-chromo alone efficiently repressed transcription in a dose-dependent manner. To confirm the functional association of MBD1-hPc2, we knocked down endogenous MBD1. In comparison to the controls, the repression of the promoter activities by GAL4-chromo was weakened after knockdown of MBD1. In addition, the expression of GAL4-fused full-length MBD1 or hPc2 alone showed stronger repression of the promoter activity (data not shown). Collectively, these data suggest that the CXXC domains of MBD1 associate with the chromodomain of hPc2.

Transcriptional Repression of HOXA Genes by MBD1 and PcG Proteins—There is a possibility that DNA methylation as well as PcG complexes is involved in the repression of *HOX* genes in mammals (40, 41), although the roles of the MBD family proteins have not been investigated. To test the roles of
MBD1 and PcG proteins in gene regulation, we performed an expression analysis of the HOXA gene cluster on human chromosome 7p15.3 using a reverse transcription-PCR method (Fig. 3A). The HOXA2, HOXA4, and HOXA7 genes were repressed in HeLa cells, whereas other HOXA genes were expressed in the cells. The expression profiles of the HOXA genes differed among the cell lines tested (supplemental Fig. 2).

To check whether the repression was related to DNA methylation, the cells were treated with 5-aza-2-deoxycytidine (5-aza-dC; 5 μM) for 3 days. Under these hypomethylation conditions, the HOXA4 and HOXA7 genes were reactivated mostly to the expression levels of the originally transcribed HOXA genes. By contrast, the HOXA2 gene seemed to be relatively resistant to derepression.

Next, we focused on characterizing the HOXA2, HOXA4, and HOXA7 genes. To test the methylation status of these HOXA gene promoters, we performed a bisulfite-based genome sequencing analysis (supplemental Fig. 3). The promoter regions of the HOXA2, HOXA4, and HOXA7 genes were highly methylated. Unexpectedly, the HOXA10 promoter was also methylated regardless of the expression state.

To check the existence of MBD1 and hPc2 in these promoters, we performed chromatin immunoprecipitation assays in HeLa cells. After formaldehyde-based cross-linking of proteins and DNAs, fragmented chromatin fractions were immunoprecipitated with anti-MBD1 or anti-hPc2 antibodies followed by PCR amplification using specific primers for the HOXA promoters (Fig. 3B). Both MBD1 and hPc2 were predominantly present in the HOXA2 promoter, whereas MBD1 was found in the HOXA4 and HOXA7 promoters. By contrast, neither MBD1 nor hPc2 was detected in the HOXA10 promoter. Last, we studied whether MBD1 and hPc2 were involved in the HOXA gene silencing (Fig. 3C). A quantitative real-time PCR analysis was performed to test the transcripts from the HOXA2, HOXA4, HOXA7, and HOXA10 genes. Compared with the controls, the levels of HOXA2 mRNA were mark-
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edly elevated by more than 3-fold after knockdown of MBD1 or hPc2. Similarly, knockdown of each protein resulted in increased levels of HOXA4 mRNA (about 2-fold) and HOXA7 mRNA (about 2–3-fold). Thus, MBD1 and hPc2 are involved in maintaining the repression of these genes. Double knockdown of both proteins did not appear to have an additional derepressive effect (data not shown). By contrast, the expression of HOXA10 did not change after knockdown of either protein. Collectively, MBD1 and hPc2 have an overlapping role in repression of the HOXA genes, and both proteins are required for maintaining the repressed state of the HOX genes.

Close Association of MBD1 and PcG Proteins in Heterochromatin Foci—To investigate the localization of MBD1 and PcG proteins in the nuclei, GFP-fused MBD1, FLAG-hPc2 and DsRed-Ring1b were expressed in HeLa cells for immunofluorescence analyses (Fig. 4A). GFP-fused MBD1 showed the formation of multiple foci in the nuclei, which were previously reported to include 4’,6-diamidino-2-phenylindole-stained heterochromatin and regions of pericentric heterochromatin in HeLa cells (11, 15). Expression of FLAG-hPc2 produced similar nuclear foci, and both MBD1 and hPc2 were colocalized at some foci (as indicated by *arrowheads*). In addition, hPc2 coexisted with Ring1b at most foci, suggesting the existence of MBD1 and PcG proteins at a part of the heterochromatin foci.

To test the localization of MBD1 deletion mutants relative to hPc2, we expressed MBD1(ΔTRD) and MBD1(MBD+NLS) nuclear localization signal (NLS)), which are shown in Fig. 1A together with FLAG-hPc2 (Fig. 4B). MBD1(ΔTRD) lacking the TRD preserved the coexistence with hPc2 at the foci. By contrast, MBD1(MBD+NLS) lacking the CXXC domains as well as the TRD formed multiple foci that were distinct from the hPc2 foci. Thus, MBD1(MBD+NLS), which is only able to bind methylated DNAs (11), lost its colocalization with hPc2.

To further check the localization of hPc2 mutants relative to MBD1, we expressed GFP-MBD1 in combination with hPc2AΔN or hPc2AΔC (shown in Fig. 1A) (Fig. 4C). hPc2AΔN lacking the chromodomain did not bind MBD1 (Fig. 1C), whereas hPc2AΔC was reported to lose the ability to form PRC1 complexes due to the absence of CtBP binding and a C-box (42, 43). hPc2AΔN showed multiple nuclear foci, but they were not colocalized with any MBD1 foci, in agreement with the data in Fig. 1C. These observations suggest that MBD1 foci are closely localized with PcG foci through the MBD1-hPc2 association. On the other hand, hPc2AΔC containing the chromodomain was unexpectedly found to be diffuse in the nuclei and lose the foci formation, suggesting that concentration of hPc2 in the foci requires the formation of PcG complexes. Taken together, our data suggest that MBD1 and PcG proteins closely exist in some heterochromatin foci through interactions between the CXXC domains and chromodomains of these proteins, respectively.

Cooperative Formation of Heterochromatin Foci by MBD1 and PcG Proteins—We then examined the localizations of endogenous MBD1 and PcG proteins in HeLa cells by immunofluorescence analysis with specific antibodies (Fig. 5A). Similar to the findings in Fig. 4, MBD1 showed multiple foci formation in the nuclei, and some foci were contained with hPc2 and Ring1b. Based on quantitative analyses of 300 foci, 60.1 and 72.7% of MBD1-containing foci coexisted with hPc2.
and Ring1b, respectively. In addition, 78.7% of hPc2-containing foci were colocalized with Ring1b. To characterize the heterochromatin foci targeted by both MBD1 and PcG proteins, we tested modifications of histone H3 using rabbit anti-trimethyl H3K9 (H3K9-3Me) and anti-trimethyl H3K27 (H3K27-3Me) polyclonal antibodies (44). MBD1 recruits H3K9 methyltransferases to CpG-methylated genomic regions (15, 17, 45). On the other hand, PRC1 complexes are localized to methyl H3K27-marked chromatin via hPc2 (28, 29). As shown in Fig. 5A, the heterochromatin foci in which MBD1 and Ring1b coexisted were stained with the trimethyl H3K9 and H3K27. About 74.7 and 27.3% of MBD1 foci appeared to be marked with H3K9-3Me and H3K27-3Me, respectively. The observations suggest that distinct types of chromatin foci, namely MBD1 foci and PcG foci, are structurally overlaid in the nuclei.

To clarify whether or not the formations of these two foci are dependent on each other in vivo, we investigated the localizations of these proteins under conditions of DNA hypomethylation (Figs. 5, B and C). The cells were cultured...
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in the presence of the DNA demethylating agent 5-aza-dC (5 μM) for 3 days. The treatment was effective for inducing DNA hypomethylation of the genome (data not shown). The use of 5-aza-dC abolished the formation of MBD1 foci marked by trimethyl H3K9, but not the foci containing hPc2 and Ring1b (upper panels). Interestingly, the number of PcG foci tended to increase 3–4-fold under conditions of DNA hypomethylation. Western blot analysis revealed that the expression levels of MBD1 and hPc2 proteins did not change in the 5-aza-dC-treated cells (supplemental Fig. 4). Next, we performed specific small interfering RNA-mediated knockdown of endogenous MBD1 or hPc2 (supplemental Fig. 1B). In the MBD1 knockdown cells, there were no foci marked by trimethyl H3K9 as well as MBD1 (Fig. 5, B and C, middle panels). By contrast, the foci containing hPc2 and Ring1b were not affected by knockdown of MBD1. Under the MBD1-depleted condition, the colocalization of Ring1b-containing foci with the trimethyl H3K27 did not significantly change (data not shown). Furthermore, knockdown of hPc2 disturbed the PcG foci detected by both hPc2 and Ring1b, whereas MBD1 and trimethyl H3K9 were found to form foci (Fig. 5, B and C, lower panels). Under the hPc2 knockdown, the colocalization of MBD1 foci with trimethyl H3K27 appeared to decrease to 10.0%, suggesting that hPc2 is involved in the colocalization of MBD1 foci and PcG foci. These data indicate that the heterochromatin foci containing MBD1 are separable from those containing PcG proteins per se. In addition, our data emphasize that MBD1 coexists with DNA methylation and trimethyl H3K9 (17, 18). As shown in Fig. 4, some of these foci appeared to be closely overlaid in the nuclei through the interaction between MBD1 and hPc2.

DISCUSSION

Mechanism of Heritable Gene Repression by MBD1 and PcG Proteins—In the present study we found that MBD1 interacts with Ring1b and hPc2, which are essential components of the maintenance PcG complex (PRC1). The overlapping roles of these proteins contribute to the transcriptional gene silencing and maintenance of heterochromatin foci formation, providing the first evidence of cooperation between MBD proteins and PcG proteins. In DNA methylation-mediated repression, MBD1 binds methyl-CpG pairs that are modified with DNMTs and recruits H3K9-specific histone methyltransferases, resulting in subsequent accumulation of heterochromatin protein 1 in the CpG-methylated regions (5, 15, 17, 45). On the other hand, EZH2 in the initiation complex (PRC2) methylates H3K27, which is then recognized by hPc2 and the other components of PRC1 for PcG-mediated repression (25–27). Thus, there are mechanistic similarities in the two major repressive pathways, especially through the involvement of both histone methylation and chromodomain proteins such as heterochromatin protein 1 and hPc2. Importantly, our knockdown of MBD1 or hPc2 revealed that these pathways cooperate in both gene silencing and heterochromatin foci formation. The model for overlapping roles of MBD1 and Polycomb group proteins in epigenetic regulation is proposed in Fig. 6. It was recently reported that EZH2 directly interacts with DNMTs to maintain CpG methylation of the EZH2 target genes (31) and that the association of BMI1 with PcG bodies requires DNMT1 (33). These findings suggest that DNMTs are associated with PRC2 and PRC1. Furthermore, the cooperation of MBD1 and the PRC1 proteins collectively emphasizes that the DNA methylation system is directly linked to the PcG system in mammals. Especially, our data revealed that both MBD1 and PRC1 proteins such as hPc2 are required for maintenance of stable repression of HOXA genes and the heterochromatin foci, suggesting their overlapping roles in epigenetic regulation. In addition, MBD1 is known to have at least five isoforms, which are alternatively spliced in the region containing the CXXC domains (11). Although the significance of the CXXC domains has remained unclear, our data that MBD1 interacts with Ring1b via CXXC1 and CXXC2 and with hPc2 via CXXC3 suggest that the MBD1 isoforms have distinct roles via interactions with the associated proteins. On the other hand, the chromodomain of hPc2 binds not only methyl-H3K27 but also the CXXC domain of MBD1 and ALL1/MLL, suggesting a new localization mechanism for the PcG proteins.

Transcriptional Control of HOXA Genes by MBD1 and PcG Proteins—We investigated the repressive roles of MBD1 and hPc2 at the HOXA gene cluster targeted by the PcG proteins and DNA methylation. Our expression studies using the DNA demethylating agent showed that the HOXA2, HOXA4, and HOXA7 genes were reactivated and
that the HOXA2 gene was relatively resistant to derepression compared with the other genes. In fact, the promoter-associated CpG islands in these genes were highly methylated. Chromatin immunoprecipitation analyses revealed that both MBD1 and hPc2 were present in the promoter regions of HOXA2, whereas MBD1 was found in the HOXA7 and HOXA4 promoters. In agreement with these data, knockdown of MBD1 or hPc2 highly reactivated HOXA2 and, to lesser extent, HOXA7 and HOXA4. Interestingly, the expressed HOXA10 gene promoter was unexpectedly CpG-methylated but not targeted by MBD1, suggesting the presence of an unknown localizing mechanism of MBD1. Thus, MBD1 and hPc2 have overlapping roles in the transcriptional silencing of the HOXA genes (Fig. 6A).

Coexistence of MBD1 and PcG Proteins in Heterochromatin Foci—In agreement with the interactions of MBD1 and PcG proteins, they appeared to be closely positioned at heterochromatin foci in the nuclei. The localizations of the deletion mutants of MBD1 and hPc2 revealed that the coexistence of these proteins at the heterochromatin foci is dependent on the CXXC domains of MBD1 and chromodomain of hPc2. Interestingly, hPc2ΔC, lacking the carboxyl-terminal region that recruits CtBP and the other components of PRC1, had a diffuse distribution in the nuclei, suggesting that the PcG foci depend on the formation of the PRC1 complex. Furthermore, the heterochromatin foci containing MBD1 and Ring1b were marked by trimethylation of H3K9 and H3K27. Importantly, the foci containing hPc2 and Ring1b were not affected by knockdown of MBD1. Similarly, the MBD1 foci did not change after knockdown of hPc2. In addition, hPc2 knockdown reduced the colocalization of MBD1 foci with the trimethyl H3K27 (27.3–10.0%), indicating that hPc2 is involved in the colocalization but not the formation of MBD1 foci. These results suggest that the MBD1 foci and PcG foci are independently assembled and that both foci are likely to be overlaid through physical interactions between MBD1 and PcG proteins (Fig. 6B). Thus, MBD1 and PcG proteins have an overlapping role in colocalization of these heterochromatin foci. In addition, the number of PcG foci significantly increased under conditions of 5-aza-dC-induced hypomethylation, suggesting the possibility that the PcG foci compensate for the decrease in CpG methylation. This may be related to the overexpression of PcG proteins in certain cancers that frequently show genome-wide DNA hypomethylation (30, 46). Luciferase reporter assays revealed that, in contrast to the CXXC domains or chromodomain, full-length MBD1 or hPc2 alone showed strong repressive activity (data not shown), suggesting that these proteins each have repressive activities and cooperate by colocalizing at heterochromatin regions. Our results shed light on the close links between MBD1 and PcG proteins for transcriptional repression and heterochromatin foci formation.

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