The Histone Methyltransferase SETDB1 and the DNA Methyltransferase DNMT3A Interact Directly and Localize to Promoters Silenced in Cancer Cells

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DNA CpG methylation can cooperate with histone H3 lysine 9 (H3-K9) methylation in heterochromatin formation and gene silencing. Trimethylation of H3-K9 by the recently identified euchromatic histone methyltransferase SETDB1/ESET may be responsible for transcriptional repression of certain promoters. Here, we show that SETDB1 associates with endogenous DNA methyltransferase activity. SETDB1 interacts with the de novo DNA methyltransferases DNMT3A and DNMT3B but not with the maintenance methyltransferase DNMT1. The interaction of SETDB1 with DNMT3A was further characterized and confirmed by in vivo and in vitro interaction studies. A direct interaction of the two proteins occurs through the N terminus of SETDB1 and the plant homeodomain of DNMT3A. Co-expression of SETDB1 and DNMT3A was essential for repression of reporter gene expression in a Gal4-based tethering assay and resulted in their recruitment to the artificial promoter. We further demonstrate that the CpG-methylated promoters of the endogenous p53BP2 gene in HeLa cells and the RASSF1A gene in MDA-MB-231 cells are simultaneously occupied by both SETDB1 and DNMT3A proteins, which provides evidence for SETDB1 being at least partly responsible for H3-K9 trimethylation at the promoter of RASSF1A, a gene frequently silenced in human cancers. In summary, our data demonstrate the direct physical interaction and functional connection between the H3-K9 trimethylase SETDB1 and the DNA methyltransferase DNMT3A and thus contribute to a better understanding of the complexity of the self-reinforcing heterochromatin machinery operating at silenced promoters.

Epigenetic gene regulation is a process that can generate heritable marks on DNA and histone N-terminal tails, which are crucial to maintain the stable patterns of gene expression. Methyltransferase activity is at the heart of the epigenetic code, where marks, such as acetylation, phosphorylation, and methylation, exert diversified and presumably more reversible effects on gene transcriptional regulation. Histone methylation occurs on both arginines and lysines, such as arginine 17 and lysine 9 of H3 that mark opposite transcription states (6). Histone H3-K9 methylation is catalyzed by members of the SET (SuVar3–9, enhancer of zeste, Trithorax) domain-containing protein family. So far, five mammalian H3-K9-specific histone methyltransferases (HMTs) have been identified, among which Suv39h1 and Suv39h2 mainly function in heterochromatic regions (7), whereas the other three HMTs, namely G9a (8), ESET/SETDB1 (9, 10), and Eu-HMTase 1 (11), mainly function in euchromatin. Interestingly, the recently identified ESET/SETDB1 is the only euchromatic HMT that can also catalyze H3-K9 triple methylation (12), which is considered as a hallmark of pericentric heterochromatin, suggesting a role of ESET/SETDB1 in connecting transcriptional silencing of gene expression and localized heterochromatin formation. ESET/SETDB1 is essential for early development (13).

DNMTs have been demonstrated to silence gene expression, at least in part through recruitment of methyl-CpG-binding domain proteins (MBDs) to CpG-methylated DNA, which interact with components of histone deacetylase (HDAC) complexes and thus recruit them to methylated DNA regions, where a compacted chromatin structure is generated (1, 14, 15). Direct interaction between DNMT3A and HDAC1 has also been reported, which supports another role of DNMT3A as a DNA methylase activity-independent corepressor (16). Similarly, ESET/SETDB1 can repress transcription 1; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; PHD, plant homeodomain.
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scription in a HMT activity-independent manner, through its interaction with HDAC1/2 (17). However, the HMT activity, such as that of Suv39h1/2, is required to generate methylated H3-K9 that is generally thought to serve as a specific binding site for heterochromatin protein 1 (HP1) (18, 19), where HP1 molecules might dimerize with each other in close vicinity and form compacted heterochromatin (20). Although extensive efforts have been made to investigate how DNA and histone methylation lead to downstream biological effects, not until recently has the central question concerning what triggers these two modifications been studied. It was found that in Neurospora (21, 22) and Arabidopsis (23), DNA methylation depends on histone methylation. In addition, Suv39h-mediated histone H3-K9 methylation was reported to direct DNA methylation at pericentric heterochromatin in mammals (24). Opposite observations that histone methylation is, directly or indirectly, controlled by DNA methylation have also been obtained in systems such as Arabidopsis (25) and in cancer cells (26, 27). However, a genetic approach unambiguously demonstrated that H3-K9 methylation at the promoter region of a tumor suppressor gene can occur independently of DNA methylation, which seems to only maintain histone methylation and gene silencing rather than initiate them (28). Although the exact interaction between DNA and histone lysine methylation systems remains unclear, studies of physical interaction between protein components of these two systems have shed light on the complexity. For example, the interaction between the methylated H3-K9-binding protein LHP1 and the CMT3 DNA methyltransferase in Arabidopsis strengthens the model that histone methylation would influence DNA methylation (23). Additionally, HP1-α associates with DNMT3B, which suggests the recruitment of DNA methyltransferase upon histone H3-K9 methylation and thereby may explain the dependence observed in Suv39h knock-out ES cells (24). Whereas HP1 protein delivers DNA methylation to chromatin marked by H3-K9 methylation, MBD proteins might recruit HMTs and trigger de novo methylation of H3-K9 at methylated DNA regions. MeCP2 associates with histone methyltransferase activity in vivo (29). MBD1 has been demonstrated to interact with SETDB1, thereby establishing a replication-coupled H3-K9 trimethylation pattern in a DNA methylation-dependent manner (27).

Here, we present evidence that an H3-K9 methyltransferase specifically interacts with de novo DNA methyltransferases. We find that SETDB1 associates with endogenous DNA methylase activity, which is most likely provided by DNMT3A, since SETDB1 interacts with DNMT3A in vitro and in vivo through its N-terminal domain but not with the maintenance methylase DNMT1. SETDB1 and DNMT3A are recruited to artificial promoters, where methylation of H3-K9 but not of DNA accompanies repressed promoter activity. Co-localization of these two epigenetic factors on the endogenous promoter regions of p53BP2 and RASSFL1A has also been observed. In summary, our results here provide a physiological and functional connection between DNA methylation and histone methylation and further support the concept of an interdependence of these two modifying enzyme systems in a self-reinforcing, circular pathway.

MATERIALS AND METHODS

Cell Culture—COS-7, HeLa, and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum.

Plasmids—The modified versions of the pcDNA3 vector, pcDNA3/Myc and pcDNA3/FLAG, have been described previously (30) and were used for expressing N-terminal Myc- or FLAG-tagged proteins in mammalian cells. Construction of plasmids pcDNA3/Myc-DNMT3A, pcDNA3/Myc-DNMT3B2, and pcDNA3/Myc-DNMT3B3 has been previously described (30). The human DNMT1 coding region was amplified by PCR from a human HeLa cDNA library (Clontech) and cloned into the EcoRI-NotI sites of pcDNA3/Myc, generating plasmid pcDNA3/Myc-DNMT1. Expression plasmids for FLAG-tagged and Gal4 DNA binding domain (Gal4DBD)-fused DNMT3A were constructed by cloning the coding region of DNMT3A from pcDNA3/Myc-DNMT3A into the EcoRI-BamHI sites of pcDNA3/FLAG and pM (Clontech), respectively. To generate GST-DNMT3A deletion mutants, DNA fragments corresponding to amino acids 1–491, 477–617, and 618–912 of DNMT3A were amplified by PCR and cloned into the EcoRI-BamHI sites of pET-GST, which has been previously described (30). For Gal4 tethering assays, luciferase reporter gene plasmids pVHL-6’×Gal4, pVHL-0’×Gal4, pGlobin-5’×Gal4, and pGlobin-0’×Gal4 were previously described (31). pCMV.FLAG-SETDB1, pCMV.FLAG-SETDB1(delta-SET) and pCMV.FLAG-SETDB1(delta-KID) were kindly provided by Dr. David C. Schultz (9). For construction of Gal4DBD-fused SETDB1 and c-Myc-tagged SETDB1, the coding region of SETDB1 was amplified by PCR and inserted into the EcoRI-Sall sites of pM and EcoRI-NotI sites of pcDNA3/Myc, respectively. For protein expression in bacteria, SETDB1 1–527 was amplified by PCR and inserted into the EcoRI-NotI sites of pcDNA3/Myc and cloned into the EcoRI-BamHI sites of pET-GST, which has been previously described (30). For Gal4 tethering assays, luciferase reporter gene plasmids pVHL-6’×Gal4, pVHL-0’×Gal4, pGlobin-5’×Gal4, and pGlobin-0’×Gal4 were previously described (31). pCMV.FLAG-SETDB1, pCMV.FLAG-SETDB1(delta-SET) and pCMV.FLAG-SETDB1(delta-KID) were kindly provided by Dr. David C. Schultz (9). For construction of Gal4DBD-fused SETDB1 and c-Myc-tagged SETDB1, the coding region of SETDB1 was amplified by PCR and inserted into the EcoRI-Sall sites of pM and EcoRI-NotI sites of pcDNA3/Myc, respectively. For protein expression in bacteria, SETDB1 1–527 was amplified by PCR and inserted into the EcoRI-NotI sites of pET-GST and EcoRI-Sall sites of pET-28a (Novagen) to obtain GST- and His-tagged recombinant proteins, respectively. All PCR-amplified sequences were verified by DNA sequencing after cloning.

Transient Transfection, Co-immunoprecipitation, Immunofluorescence, and Luciferase Assays—Transfection was performed largely as described previously (32). In brief, COS-7 cells were transiently transfected with transfection reagent F-2 (Targeting Systems) in 100-mm plates for co-immunoprecipitation, 6-well plates for immunofluorescence, or 12-well plates for luciferase reporter assays. For co-immunoprecipitation, ~48 h after transfection, cells were scraped into radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and protease inhibitor mixture tablets (Roche Applied Science)), followed by centrifugation at 12,000 rpm for 30 min in an Eppendorf centrifuge. Cell lysates were then cleared with protein A/G beads (Santa Cruz Biotechnology) for 1 h at 4 °C. 1 μg of mouse anti-FLAG antibody (Sigma) was added to the cell lysates and incubated overnight at 4 °C on a rotator. 30 μl of protein A/G beads were added and incubated for another 3 h. For co-immunoprecipitation between endogenous SETDB1 and DNMT3A proteins (endogenous co-IP),
fetal mouse gonads were obtained at 15.5 days postcoitum and trypsinized. Somatic gonadal cells were obtained as a green fluorescent protein-negative cell population by flow sorting (33). Single cell suspensions were then washed with phosphate-buffered saline, and cell lysates were prepared as described above but incubated with rabbit anti-ESET/SETDB1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) followed by incubation with protein A/G beads. Beads were washed three times with radioimmune precipitation buffer and subjected to SDS-PAGE. Blots were probed with rabbit anti-c-Myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1 μg/ml of blocking buffer (5% nonfat milk in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.1% Tween 20)). 5% of cell extracts were also subjected to immunoblotting with either anti-FLAG at 1 μg per 2 ml or anti-c-Myc at 1 μg per 20 ml of blocking buffer to check the expression levels of the proteins. Appropriate horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used at 1:1,000 and 1:2,000 dilution, respectively.

DNA Methylation Assays—DNA methylation was determined in E. coli Rosetta (DE3)pLysS (Novagen) and by bisulfite sequencing. For DNA methylation assays, cultures of E. coli were grown in LB medium supplemented with 100 μg/ml ampicillin and galactose at 37 °C to an OD600 of 0.1. Cells were then harvested by centrifugation and lysed with chloroform and phenol. DNA was recovered from the aqueous phase. Bisulfite-treated DNA samples were amplified by PCR using specific primers. PCR products were bidirectionally sequenced by the local DNA sequencing facility. The resulting sequences were analyzed for methylation status using MethPrimer software (38), spanning either the six repetitive promoter regions or the VHL promoter region upstream of the luciferase gene: 5’-GGTGTGGAGGATTTTTGG-TAAGTGTA-3’ (forward) and 5’-CAAAAAATCTCCTCAA-CACC-3’ (reverse) or the VHL promoter region upstream of the luciferase gene: 5’-GATGTGGAGGTGTTTTTTTGTG- TCTGTA-3’ (forward) and 5’-ATCTCTTCATAACCTTATAACTAC-TTCC-3’ (reverse). Clones containing PCR products were produced using the TA cloning kit (Invitrogen) and sequenced at the local DNA sequencing facility.

DNMT Magnetic Beads Assay—DNMT activity was tested in the magnetic beads assay essentially as described previously (35) with the difference that DNMT activities were directly or indirectly obtained by immunopurifying overexpressed c-Myc-tagged proteins in COS-7 cells, using the Catch and Release kit (Upstate Biotechnology). Biotinylated double-stranded DNA with the previously reported sequence (35) was synthesized at the City of Hope DNA synthesis facility and used as substrate for methylation reactions in the presence of tritium-labeled S-adenosylmethionine (Amersham Biosciences) in Eppendorf tubes. All magnetic separations were performed in Eppendorf tubes in a magnetic rack (Dynal), and tritium incorporation was measured by scintillation counting.

GST Pull-down Assay—GST fusion proteins were produced in Escherichia coli Rosetta (DE3)pLysS (Novagen) and bound to glutathione-Sepharose-4B beads (GE Healthcare). Hexahistidine SETDB1 1–527 was purified with His-Bind Buffer Kit (Novagen) according to the manufacturer’s instructions. Eluted SETDB1 was dialyzed against storage buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, 50% glycerol). Nontransfected COS-7 cell lysates or lysates containing overexpressed c-Myc-DNMT1, c-Myc-DNMT3A, or c-Myc-SETDB1 were prepared as described above. 1–4 μg of glutathione-Sepharose beads-bound GST fusion proteins were incubated at 4 °C for 3 h with either cell lysates or 1 μg of His-SETDB1 1–527 in a 500-μl total volume of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 3 mM MgCl2, 0.1% Triton X-100, 5% glycerol, and 25 mg/ml bovine serum albumin) on a rocking platform. 25 μl of cell lysates or 10 ng of His-tagged protein were set aside as input. Pelleted Sepharose beads were washed four times with binding buffer containing 250 mM NaCl and fractionated on 10% SDS-PAGE. Nitrocellulose membranes (Bio-Rad) were probed with anti-c-Myc, anti-DNMT1 (Santa Cruz Biotechnology), anti-DNMT3A (Santa Cruz Biotechnology), or anti-His (GE Healthcare) antibodies and subjected to auto fluorography.

Bisulfite Sequencing—Reporter DNA was recovered from COS-7 cells 24 h after transfection, using an alkaline extraction method (36). Sodium bisulfite overnight treatment of the recovered DNA was performed as previously described (37). Two pairs of PCR primers were designed using the online MethPrimer software (38), spanning either the six repetitive Gal4 binding sites 5’-GTAAATAGGGTTGTGTTTATG- TAAATGTA-3’ (forward) and 5’-CAAAAAATCTCCTCCTAAAC- CACC-3’ (reverse) or the VHL promoter region upstream of the luciferase gene: 5’-GGTGTGGAGGAATTTTTTTGG-TAAGTGTA-3’ (forward) and 5’-ATCTCTTCATAACCTTATAACTAC-TTCC-3’ (reverse). Clones containing PCR products were produced using the TA cloning kit (Invitrogen) and sequenced at the local DNA sequencing facility.

Chromatin Immunoprecipitation (ChIP) and Reporter Co-IP—ChIP assays were performed largely as described previously (39). In brief, HeLa or MDA-MB-231 cells were grown in 150-mm dishes. Cells were fixed with formaldehyde, and chromatin in nuclear lysis buffer was sheared using a sonicator (Misonix). The resulting supernatant contained 200–1,000-bp-long DNA fragments. Immunoprecipitation was conducted with 2 μg of normal rabbit IgG (Santa Cruz Biotechnology), anti-HDAC1 (Santa Cruz Biotechnology), anti-DNMT3A, anti-SETDB1 (Upstate Biotechnology), or anti-trimethylated H3-K9 (Abcam) and 30 μl of preblocked protein A/G Plus-agarose with rotation overnight at 4 °C. Immunocomplexes were washed and eluted from beads. In sequential ChIP experiments, the initial immunoprecipitated complexes were eluted by incubation with 10 mM dithiothreitol at 37 °C for 30 min and diluted 1:50 in IP dilution buffer. Eluates were then reimmunoprecipitated with second antibodies (38). PCR amplification was performed with 2 μl of (1:25) DNA using 35–42 cycles. The following primers were used: RASSFIA (residues 11–431), 5’-AGGCCCTAGC- TCATTGAGCT-G-3’ (forward) and 5’-AGTGTGAGTGG- ACAAGGAT-3’ (reverse); p53BP2 (residues –189 to +12), 5’-AAAAGGAAAAGGGCGGCG-3’ (forward) and 5’-GAGGGTCGGGGTCCCTC-3’ (reverse); β-actin coding region (residues 68–327) (39), 5’-CTTACCATAAGGAT- GATATAGC-3’ (forward) and 5’-ATTITCTCCATGTCG- TCCAGTTG-3’.
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In reporter co-IP assays, to test the recruitment of SETDB1 to the artificial VHL promoter containing 6×Gal4 response elements through the Gal4-DNA binding domain-fused DNMT3A, or vice versa, COS-7 cells were transiently transfected with p6×Gal4-VHL-luciferase or p0×Gal4-VHL-luciferase reporter plasmid with pM-DNMT3A and pCMV.FLAG-SETDB1 or pM-SETDB1 and pcDNA3/FLAG-DNMT3A. Soluble chromatin fraction was prepared and immunoprecipitated. Anti-dimethyl-H3-K9, and anti-acetylated H3-K9 antibodies were obtained from Upstate Biotechnology. PCR was performed as described above. The following primers, located on the pGL3-luciferase backbone spanning the VHL promoter and six Gal-4 response elements were used: 5′-CAAGTGCGAGTGCAGAGAACA-3′ (forward) and 5′-ACCAGGGCGTATCCTTTCAT-3′ (reverse).

RESULTS

SETDB1 Associates with DNMT Activity—The H3-K9 trimethylase ESET/SETDB1 interacts with HDAC1 (17) and can repress euchromatic gene expression through methylated H3-K9 bound HP1 (40). It has also been reported that ESET/SETDB1-mediated H3-K9 methylation is dependent on MBD1 and inherited through DNA replication to help maintain stable heterochromatin at methylated DNA, suggesting a DNA methylation-directed H3-K9 trimethylation process (27). Since ESET/SETDB1 can interact with various components of the heterochromatin maintenance machinery, such as HDAC1/2, HP1 (41, 42), and MBD1 (27), it was intriguing to test whether ESET/SETDB1 could also directly associate with DNMT activity. To test this possibility, SETDB1 as well as DNMT1 and DNMT3A were overexpressed in COS-7 cells and immunoprecipitated for an in vitro DNMT magnetic bead assay. Immunoprecipitated exogenous DNMT3A (Fig. 1, lane 3) showed 1.6-fold higher activity over empty vector (Fig. 1, lane 1), whereas immunoprecipitated exogenous DNMT1 showed 4.8-fold enhancement over the control, which is consistent with previous reports using unmethylated double-stranded DNA as substrates (35). SETDB1-retained endogenous DNMT activity (Fig. 1, lane 4) was comparable with that of exogenous DNMT3A, suggesting that SETDB1 is associated with endogenous DNMT proteins.

SETDB1 Specifically Associates with DNMT3 Proteins—Among the four DNMTs identified to date, DNMT1 maintains the DNA methylation pattern through replication, whereas DNMT3A and DNMT3B are primarily de novo DNA methylases. In COS-7 cells, endogenous DNMT1 has a relatively strong expression level, whereas the DNMT3A protein level is relatively low but detectable (see Fig. 3B, lanes 7 and 10). Using exogenous DNMT3B2 as a positive control in immunoblotting assays, no detectable expression of endogenous DNMT3B was observed in COS-7 cells (supplemental Fig. S1B), which suggests that the SETDB1-associated DNMT activity shown in Fig. 1 was from DNMT1 and/or DNMT3A. Immunofluorescence assays were initially performed to assess potential co-localization patterns between SETDB1 and DNMTs in COS-7 cells (Fig. 2A). The experimental setting was first validated by testing the co-staining of DNMT3A and HDAC1 (supplemental Fig. S1A, a–d), proteins known to interact with each other (16). Using rabbit anti-SETDB1 antibody, we next observed that the endogenous SETDB1 signal distributed throughout euchromatin (Fig. 2A, b and f), which confirmed the SETDB1 localization pattern reported elsewhere (9, 40, 42). Either FLAG-DNMT1 or FLAG-DNMT3A was then introduced into COS-7 cells for co-immunostaining, performed with rabbit anti-SETDB1 and mouse anti-FLAG antibodies. Pearson’s correlation and overlap coefficient, two well defined and commonly accepted means for describing the extent of overlap between image pairs, were calculated (for Fig. 2A d), Pearson’s correlation $R_r = 0.81 ± 0.03$, overlap coefficient $r = 0.89 ± 0.03$, $n = 8$ cells; for Fig. 2A h, Pearson’s correlation $R_r = 0.87 ± 0.01$, overlap coefficient $r = 0.91 ± 0.02$, $n = 8$ cells), which suggests partial co-localization between endogenous SETDB1 and exogenous DNMTs.

To identify the DNMT responsible for SETDB1-associated DNA methylation activity, DNMT1 and DNMT3A as well as
SETDB1 were overexpressed in COS-7 cells, and the interactions were tested in co-immunoprecipitation assays. Two DNMT3B alternative splicing variants, DNMT3B2 and DNMT3B3, were also included in the assay. Both c-Myc-tagged DNMTs (Fig. 2, lanes 1–4) and FLAG-tagged SETDB1 (lanes 17–20) were expressed equally well in COS-7 cells. When various DNMTs were expressed alone, anti-FLAG antibody did not co-immunoprecipitate any DNMT protein (lanes 5–8 and 13–16). However, in the presence of exogenously expressed FLAG-SETDB1, a strong co-immunoprecipitated DNMT3A band and a relatively weaker DNMT3B2 band were observed in the immunoprecipitates of anti-FLAG antibody (lanes 10 and 11). No DNMT1 was detected in the immunocomplex (lane 9), suggesting that DNMT3s (DNMT3A and DNMT3B2) instead of DNMT1 associate with SETDB1. Interestingly, DNMT3B3, an enzymatically inactive splicing variant, also interacts with SETDB1 (lane 12). Since DNMT1 cannot interact with SETDB1 and DNMT3B expression cannot be detected in COS-7 cells (supplemental Fig. S1B), it is reasonable to conclude that the DNMT activity co-immunoprecipitated by SETDB1 (Fig. 1, lane 4) was provided by endogenous DNMT3A. We therefore decided to focus on DNMT3A for the following biochemical studies.

To further investigate whether endogenous DNMT3A and SETDB1 could form a complex under physiological conditions, co-immunoprecipitation assays were conducted in fetal mouse somatic gonadal cells, where the level of full-length DNMT3A protein is relatively high (Fig. 2C) (43, 44). Whereas no DNMT3A was immunoprecipitated by normal rabbit IgG (lane 2), anti-SETDB1 antibody specifically co-immunoprecipitated endogenous DNMT3A (Fig. 2C, lane 3). Taken together, these results indicate a specific in vivo interaction between SETDB1 and DNMT3A.

The SETDB1 N-terminal Domain Directly Interacts with the DNMT3A Plant Homeodomain (PHD)—Although SETDB1 could associate with DNMT3A in COS-7 cells, it was possible that the interaction is mediated through endogenous bridging proteins such as HDAC1 or HP1. We therefore asked whether these two proteins could directly bind to each other. We first sought to define the responsible region of SETDB1 for interaction with DNMT3A, by testing fragments of SETDB1 in a similar co-immunoprecipitation assay. COS-7 cells were co-transfected with vectors expressing FLAG-tagged full-length SETDB1 or SETDB1 deletion mutants (amino acids 1–951 and 570–1291) and vectors expressing c-Myc-tagged DNMT1 or DNMT3A. Potential complexes were precipitated with an antibody against the FLAG epitope and examined with anti-c-Myc antibody. Anti-FLAG antibody efficiently immunoprecipitated the full-length FLAG-SETDB1 protein (Fig. 3A, lane 13) and co-immunoprecipitated DNMT3A (lane 9). Although SETDB1 1–951 and

**FIGURE 2. SETDB1 specifically associates with DNMT3s in vivo.** A, FLAG-DNMT3A partially colocalizes with SETDB1. COS-7 cells grown on coverslips were transfected with 2 μg of pcDNA3/FLAG-DNMT1 (a–d) or DNMT3A (e–h) in 6-well plates. Rabbit anti-SETDB1 (b and f) antibodies were used for co-immunostaining with mouse anti-FLAG antibodies (c and g). Dye-conjugated goat anti-rabbit antibody labeled endogenous SETDB1 (Fig. 1a). Anti-FLAG antibody specifically co-immunoprecipitates endogenous DNMT3A (Fig. 2C, lane 3). Taken together, these results indicate a specific in vivo interaction between SETDB1 and DNMT3A.

B, SETDB1 specifically interacts with DNMT3s. COS-7 cells were co-transfected with 6 μg of each c-Myc-tagged DNMT expression construct, and with 4 μg of either pcMV.FLAG vector or pcMV.FLAG-SETDB1. Cell extracts were prepared 48 h after transfection and immunoprecipitated (IP) with 2 μg of anti-FLAG antibody (lanes 5–12). Immunoprecipitates were analyzed on an 8% SDS-polyacrylamide gel and immunoblotted (IB) with anti-c-Myc antibody. Protein complexes were precipitated with an antibody against the FLAG epitope and examined with anti-c-Myc antibody. Anti-FLAG antibody efficiently immunoprecipitated the full-length FLAG-SETDB1 protein (Fig. 3A, lane 13) and co-immunoprecipitated DNMT3A (lane 9). Although SETDB1 1–951 and
SETDB1 and DNMT3A

FIGURE 3. The N terminus of SETDB1 directly interacts with the PHD of DNMT3A. A, the N terminus of SETDB1 specifically associates with DNMT3A. COS-7 cells were co-transfected with constructs (5 μg each) expressing full-length FLAG-tagged SETDB1 or fragments and constructs (5 μg each) expressing c-Myc-tagged DNMT1 or DNMT3A, as indicated. Expression of SETDB1 fragments was assayed by immunoblotting using anti-FLAG antibody (lanes 1–14). Consistent with reports by other researchers (9), nonspecific bands were also detected. Each immunoprecipitation was performed with 2 μg of anti-FLAG antibody, and immunoprecipitates were subjected to 8% SDS-PAGE and blotted with anti-c-Myc antibody (lanes 2–5 and 7–10). 5% of cell lysates was loaded as input (lanes 1 and 6). B, the N terminus of SETDB1 specifically interacts with DNMT3A in vitro. GST and GST-fused SETDB1 1–527 were purified from E. coli and visualized by Coomassie Blue staining (top). 2 μg of matrix-bound GST recombinant proteins were mixed with cell lysates containing either exogenous c-Myc-tagged proteins (lanes 1–6) or endogenous proteins (lanes 7–12), followed by incubation at 4 °C for 3 h. Proteins bound to beads were eluted and analyzed by immunoblotting with anti-c-Myc, anti-DNMT1, or anti-DNMT3A antibodies. C, the PHD of DNMT3A interacts with SETDB1. DNMT3A fragments were purified as GST fusion proteins from bacteria and visualized by Coomassie Blue staining (top). Lysates from c-Myc-SETDB1 transfected cells were mixed with protein-bound beads and retained proteins were analyzed with anti-c-Myc antibody (bottom). D, the N terminus of SETDB1 directly interacts with the PHD of DNMT3A. His-tagged SETDB1 1–527 and GST-tagged DNMT3A 477–617 were observed between DNMT1 and any of the SETDB1 fragments (lanes 1–4), confirming the specific binding of SETDB1 to DNMT3A.

To further test whether the SETDB1 N-terminal fragment is sufficient for the binding to DNMT3A, a GST pull-down assay was performed, in which GST agarose-bound SETDB1 1–527 was mixed with COS-7 cell lysates containing either overexpressed c-Myc-tagged or endogenous DNMT3A. Binding specificity was also tested in a similar setting using DNMT1 as a negative control (Fig. 3B, lanes 1–3 and 7–9). Whereas no interaction or background interaction was observed between GST and DNMT3A, GST-fused SETDB1 1–527 (Fig. 3B, staining panel) strongly bound to both exogenous and endogenous DNMT3A (lanes 4–6 and 10–12), indicating that amino acids 1–527 of SETDB1 are responsible for the recruitment of SETDB1 to DNMT3A. We next sought to identify the region in DNMT3A responsible for the interaction. Structurally, DNMT1, -3A, and -3B can be divided into two domains, the highly conserved C-terminal region containing catalytic motifs and the less conserved N-terminal regulatory domains that, in the case of DNMT3A and DNMT3B, are characterized by a PWPP motif and a PHD finger-like domain. Three different DNMT3A fragments were therefore fused to GST: DNMT3A amino acids 1–491, containing the PWPP motif; DNMT3A amino acids 477–617, containing the PHD, and DNMT3A amino acids 618–912, containing the catalytic C-terminal domain. Concentrations of these GST fusion proteins were assessed by Coomassie Blue staining (Fig. 3C, staining panel), and similar amounts of each protein bound to beads were then observed between DNMT1 and any of the SETDB1 fragments (lanes 1–4), confirming the specific binding of SETDB1 to DNMT3A.

SETDB1 570–1291 were expressed and immunoprecipitated similarly (lanes 11 and 12), a strong DNMT3A signal was only detected in the immunoprecipitates of SETDB1 1–951 (lanes 7 and 8), suggesting that the N-terminal fragment of SETDB1 was required for the interaction with DNMT3A. No interaction was observed between DNMT1 and any of the SETDB1 fragments (lanes 1–4), confirming the specific binding of SETDB1 to DNMT3A.
Since recombinant full-length proteins were technically unavailable from bacteria and responsible domains for interaction were already identified, we next tested a direct interaction between SETDB1 and DNMT3A, in a cell-free system using protein fragments. Both recombinant His-tagged SETDB1 1–527 and GST-tagged DNMT3A 477–617 were purified from E. coli (Fig. 3D, staining panel) and used in a GST pull-down assay. Whereas GST-agarose alone displayed no binding signal, a strong interaction between GST-DNMT3A 477–617 and His-SETDB1 1–527 was observed, in a solution presumably without any other potential bridging proteins, which suggests a direct interaction between the histone methylase SETDB1 and the DNA methylase DNMT3A. Recently, a direct interaction between the SETDB1 N-terminal domain and the PHD of KAP1, a corepressor for KRAB zinc finger proteins, has also been observed (9). PHDs are known to mediate protein-protein interactions. Thus, the SETDB1–DNMT3A interaction shown here is another example of binding between the SETDB1 N-terminal region and a PHD, suggesting that the SETDB1 N-terminal part may serve as a PHD recognition motif.

**SETDB1 and DNMT3A Form a Repressive Functional Complex on Artificial Promoters**—Since we have demonstrated that SETDB1 is associated with DNMT3A in vivo, it was intriguing to test whether this association may be functional for gene regulation. Neither SETDB1 nor DNMT3A is a DNA sequence-specific binding protein per se. We therefore tested this idea in a tethering assay, in which either protein was fused with a Gal4 DNA binding domain and co-introduced into cells with luciferase reporters containing Gal4 binding sites. Once the assay system was set up, the other protein was tested for its transcriptional regulation function. Gal4-fused DNMT3A and SETDB1 were both able to repress the activity of an artificial VHL promoter that includes six repetitive Gal4 binding sites in a dose-dependent manner (supplemental Fig. S2, A and B; Fig. 4, A (lanes 1 and 2) and B (lanes 1 and 2)). Interestingly, with increasing amounts of co-transfected SETDB1, the Gal4-DNMT3A-repressive activity was further potentiated about 2-fold (Fig. 4A, lanes 3 and 4). A 4-fold enhancement of reporter gene repression was observed for Gal4-SETDB1 when DNMT3A was co-expressed (Fig. 4B, lanes 3 and 4), which suggests that the indirect recruitment of secondary co-repressors such as FLAG-SETDB1 or c-Myc-DNMT3A to the VHL promoter were through interactions with Gal4DBD-fused DNMT3A or SETDB1, respectively. To test whether the function of the SETDB1–DNMT3A complex is promoter-dependent, we performed similar reporter gene assays in the context of an α-globin gene promoter artificially conjugated with five repetitive Gal4 binding sites, and we observed similar repression patterns (supplemental Fig. S2, C and D). Reporter genes with the same promoter backbone but which lack Gal4 binding sites were also tested, and no repression was observed in similar transfection experiments (Fig. 4, C and D). Taken together, these data demonstrate a mutual facilitation between SETDB1 and DNMT3A in repression function upon binding to promoters and suggest the formation of a universal repressor complex between these two proteins.

Since SETDB1 and DNMT3A seem to function together to repress reporter genes, we next tested whether both proteins are actually recruited to the promoter region of the reporter gene, one protein being directly recruited through the Gal4 DNA binding domain and the other being indirectly recruited through protein-protein interactions. DNMT3A methylates DNA, and SETDB1 methylates H3-K9, and both proteins can directly interact with HDAC1 and can display enzymatic activity-independent repression functions. To differentiate these activities potentially responsible for the observed repression of the artificial VHL promoter, we analyzed the DNA methylation and histone H3 methylation status as well as HDAC1 recruitments to this promoter (Fig. 5). To test the DNA methylation status, the reporter plasmid VHL-6×Gal4-luciferase was first introduced into COS-7 cells with Gal4DBD-fused DNMT3A and then recovered from cells and subjected to bisulfite sequencing. None of the 57 CpGs in either the region containing six repetitive Gal4 binding sites or the VHL pro-

![Figure 4](image-url)
SETDB1 and DNMT3A

**A**
- p6xGal4-VHL-luciferase: 532 bp
- Luciferase

**B**
- a: p6xGal4-VHL-luciferase: 532 bp
- b: p0xGal4-VHL-luciferase: 414 bp
- ChIP on reporters
  - Input
  - IgG
  - Gal4
  - me2K9
  - me3K9
  - acK9

**C**
- a: 532 bp
- b: 414 bp
- Reporter (a or b) + Gal4-DNMT3A + Flag-SETDB1

**D**
- a: 532 bp
- b: 414 bp
- Reporter (a or b) + Gal4-DNMT3A + Flag-DNMT3A

**FIGURE 5.** SETDB1 and DNMT3A are recruited to the promoter region of transiently transfected Gal4 binding sites containing VHL-luciferase. **A**, schematic diagrams of the VHL promoter containing reporter genes with (a) and without (b) six repetitive Gal4 binding sites. Positions of the PCR primers are indicated as pairs of opposing arrows in the diagram. **B**, accompanying H3-K9 trimethylation with SETDB1 recruitment to the VHL promoter. COS-7 cells were transfected with either Gal4DBD (c) or Gal4-SETDB1 (d) expression constructs (4 µg each) and pVHL-6×Gal4-LUC (4 µg) or Gal4-SETDB1 and pVHL-0×Gal4-LUC (4 µg each). After 48 h, cells were fixed with formaldehyde and harvested. Immunoprecipitation of sheared chromatin was performed with 1 µg of each indicated antibody, and the precipitated DNA was analyzed by PCR using primers that flank both Gal4 binding sites and the VHL promoter region. Results shown were obtained with a 1:100 dilution of input and precipitated DNA and are representative of three independent experiments. **C**, recruitment of Flag-SETDB1 and endogenous HDAC1 to the promoter region through Gal4DBD-fused DNMT3A. **D**, recruitment of Flag-DNMT3A and endogenous HDAC1 to the promoter region through Gal4DBD-fused SETDB1. Reporter co-IP assays were performed in cells transfected with 2 µg of reporters containing Gal4 binding sites (a) or not (b) as well as 4 µg of constructs expressing Gal4 fusion proteins and FLAG-tagged proteins as indicated. Precipitated DNA was analyzed by PCR using the same set of primers. The PCR product containing Gal4 binding sites was displayed as a 532-bp band on the 2% agarose gel, and the 414-bp band represents the region without Gal4 binding sites. Results shown are representative of three independent experiments.

moter region was methylated (data not shown) after 24 h, suggesting that Gal4DBD-fused DNMT3A, despite its physical interaction with the promoter region (Fig. 5C), could not methylate the VHL promoter. The repression function of DNMT3A observed in the reporter gene assay might thus solely come from endogenous DNMT3A binding co-repressors, such as HDAC1, in this particular experimental setting.

To test the histone H3 lysine 9 methylation status on the artificial promoter region, a reporter co-IP assay was performed in COS-7 cells, in which the reporter plasmid 6×Gal4-VHL-luciferase was first introduced into COS-7 cells with either Gal4DBD alone or Gal4DBD-fused SETDB1. Immunoprecipitates using antibodies against dimethylated H3-K9, trimethylated H3-K9, or acetylated H3-K9 were then obtained, and DNA from immunoprecipitates was analyzed by PCR using primers for sequences on the backbone of the pGL3-basic luciferase plasmid. These primers produce a 532-base pair PCR product when flanking the six repetitive Gal4 binding sites and the VHL promoter and a 414-base pair PCR product when there are no Gal4 binding sites in the reporter plasmid (Fig. 5A). Anti-Gal4 antibody could associate with DNA fragments containing Gal4 binding elements, indicating occupancy of this VHL promoter adjacent region by either Gal4DBD alone or Gal4DBD-SETDB1 (Fig. 5B, lanes 5 and 6). Whereas normal rabbit IgG only precipitated background level of reporter plasmid, antibodies against dimethylated H3-K9 weakly and antibodies against trimethylated H3-K9 substantially enriched DNA fragments that were occupied by Gal4-SETDB1 (Fig. 5B, lanes 7–10), suggesting that methylation of histone H3 lysine 9 in the vicinity of Gal4 binding sites is specifically mediated by SETDB1. Reporters without Gal4 elements were also tested; however, neither SETDB1 recruitment nor histone H3 methylation was observed using the same sets of primers (Fig. 5B, lanes 15–17), confirming that histone modification is dependent on the recruitment of a responsible enzyme to the promoter region. Interestingly, H3-K9 acetylation was also detected (lanes 11, 12, and 18), which might be responsible for the relatively high basal activity of the reporter; additionally, this transcriptionally active mark was clearly diminished when SETDB1 was bound (compare lanes 11 and 12). SETDB1 interacts with HDAC1 through its N-terminal domain and can inhibit euchromatic gene expression through the histone deacetylase complex (17). Therefore, our data also demonstrate the deacetylation process resulting from indirect recruitments of HDAC. Co-transfection of multiple plasmids might not guarantee simultaneous uptake of all vectors by cells; therefore, the remaining acetylation signal in lane 12 might be due to the absence of SETDB1 in particular cell populations instead of incomplete SETDB1-HDAC-mediated histone deacetylation. Reporter co-IP assays were also performed in COS-7 cells transfected in a similar setting as in the luciferase assay shown in Fig. 4. A and B. Similar signals were amplified from input chromatin (i.e. before immunoprecipitation). However, PCR analysis of DNA immunoprecipitated by antibodies against Gal4-DNMT3A (anti-Gal4), Flag-SETDB1 (anti-FLAG), and endogenous HDAC1 (anti-HDAC1) indicated that the Gal4 response elements containing reporter were significantly enriched in the immunoprecipitates, compared with the
reporter lacking Gal4 binding sites (Fig. 5C). Normal rabbit IgG precipitated neither reporter plasmid. Therefore, the results demonstrate that FLAG-SETDB1 was specifically recruited to the Gal4 response elements, most likely through Gal4-fused DNMT3A, considering the fact that SETDB1 can directly interact with DNMT3A (Fig. 3). Reporter co-IP experiments were also conducted in which SETDB1 instead was fused to Gal4DBD as the primary DNA binding protein, and similar recruitments of FLAG-DNMT3A and endogenous HDAC1 were also observed (Fig. 5D). These results imply that the observed co-repressor activity of either SETDB1 or DNMT3A when not fused to Gal4DBD in reporter assays (Fig. 4) is due to their indirect recruitments to the promoter through their Gal4DBD-fused binding partners, Gal4-DNMT3A or Gal4-SETDB1, respectively. In addition, endogenous HDAC1 was also indirectly recruited to the promoter region in the 6×Gal4-VHL reporter in both settings (Fig. 5, C and D), which thus provided further evidence of the interaction between HDAC1 and DNMT3A or SETDB1. Since both HDAC1 and DNMT3A (Fig. 3D) can directly bind to SETDB1, it is interesting to test whether these three proteins could form a complex. Lysates of COS-7 cells overexpressing all three proteins were used for co-immunoprecipitation assays, and both HDAC1 and DNMT3A were simultaneously detected in SETDB1 immunoprecipitates, which suggests that associations of HDAC1 and DNMT3A with SETDB1 are not mutually exclusive ( supplemental Fig. S3). Taken together, this finding confirms and expands the model developed from the reporter gene activity assays, showing that the interaction between SETDB1 and DNMT3A is functional in repressing gene expression, in which the repression mark of histone H3-K9 trimethylation can be generated, and HDAC1 can be recruited.

**SETDB1 and DNMT3A Colocalize on Endogenous Promoters.—** Since mutual recruitments of SETDB1 and DNMT3A to an artificial promoter were observed, we next checked whether these two proteins could colocalize on certain endogenous promoters. The promoter of p53BP2 was recently identified as one of the SETDB1 targets in a ChIP-cloning procedure (27). We therefore tested the potential recruitment of DNMT3A to the p53BP2 promoter in HeLa cells, assuming that SETDB1 may be the docking protein. Consistent with the original report, SETDB1 was associated with the p53BP2 promoter (Fig. 6A, lane 4). Whereas normal rabbit IgG did not precipitate any DNA (lane 2), a significant amount of PCR products representing the p53BP2 promoter region was obtained from immunoprecipitates by anti-DNMT3A antibody (lane 3), indicating occupancy of the p53BP2 promoter by DNMT3A and SETDB1. Control ChIP assays did not show any significant recruitment of these two proteins to the β-actin coding region (Fig. 6A, bottom panel). The specific recruitment of DNMT3A to the normally hypermethylated p53BP2 promoter in HeLa cells (27) suggests that the de novo DNA methylase DNMT3A might also function in DNA methylation maintenance as a “proofreader,” possibly by methylating the hemimethylated CpG sites missed by DNMT1. In addition, SETDB1 can be recruited to the p53BP2 promoter region through methylated DNA-binding protein MBD1 and thus generates an inheritable H3-K9 trimethylation pattern in a

![Image](https://example.com/image.png)

**FIGURE 6.** SETDB1 and DNMT3A are recruited to promoter regions of the endogenous p53BP2 and RASSF1A genes. A, ChIP assay with the endogenous p53BP2 promoter in HeLa cells. Sheared chromatin was prepared from HeLa cells, and the ChIP procedure was performed with 2 μg of each indicated antibody. The precipitated DNA was analyzed by PCR using primers that amplify either the p53BP2 promoter region (residues 189 to +12) (top) or the β-actin coding region (residues 68–327) (bottom). Results were obtained with 2 μl of precipitated DNA and are representative of three independent experiments. B, ChIP assay with the endogenous RASSF1A promoter in MDA-MB-231 cells and HeLa cells. Sheared chromatin was immunoprecipitated with 2 μg of each indicated antibody. PCR analysis of precipitated DNA was performed with primers that amplify either the p53BP2 promoter region (residues 68–327) (middle) or the RASSF1A 5′ region (residues 11–431) (top for MDA-MB-231 cells and bottom for HeLa cells). RASSF1A is silenced in MDA-MB-231 cells but active in HeLa cells (37). C, sequential ChIP assays to test simultaneous recruitments of SETDB1 and DNMT3A. Cross-linked immunocomplexes from primary ChIPs were eluted and subjected to precipitation using a different antibody. PCR analysis was performed with primers targeting the same RASSF1A region. Results shown are representative of two independent experiments.

DNA methylation-dependent manner; therefore, the observed DNMT3A recruitment associated with that of SETDB1 (Fig. 6A) also suggests a self-reinforcing HMT-DNMT network that maintains heterochromatin formation.

Besides the known SETDB1-regulated p53BP2 promoter, we also tested the potential co-localization of SETDB1 and
SETDB1 and DNMT3A

DNMT3A on the upstream region of RASSF1A, a tumor suppressor gene. Although a high frequency of methylation of the RASSF1A gene has been found in various cancers (45), mechanisms underlying promoter hypermethylation in pathological conditions still remain elusive. Recently, the occurrence of H3-K9 trimethylation at this promoter region has been reported to precede DNA methylation at early passages of human mammary epithelial cells, implying a role of the responsible HMT in initiating an inactive chromatin state at the RASSF1A promoter (46). Among the five H3 lysine 9 methyltransferases identified in mammals, ESET/SETDB1 is known to possess H3-K9 trimethylation HMT activity. A ChIP assay was then performed in MDA-MB-231 cells, where the RASSF1A promoter is hypermethylated (37), using antibodies against SETDB1, DNMT3A, HDAC1, and trimethylated H3-K9 (Fig. 6B). Consistent with the previous finding, H3-K9 trimethylation was detected at the 5′ region of RASSF1A exon 1, which is part of the CpG island (Fig. 6B, lane 6). Whereas normal rabbit IgG did not precipitate this region (lane 2), strong association with this region of SETDB1, DNMT3A, and HDAC1 was observed (lanes 3–5). Immunoprecipitates were also examined for the presence of actin coding region sequences (Fig. 6B, middle), and control ChIP assays using primers against RASSF1A exon 1 were also conducted in HeLa cells (Fig. 6B, bottom). The β-actin and RASSF1A genes are actively expressed in the latter scenarios, and the same antibodies failed to precipitate either DNA fragments. The evidence provided here suggests that SETDB1 might be at least partially if not completely responsible for the observed H3-K9 methylation. Additionally, DNMT3A and HDAC1 were also detected at the 5′ region of the endogenous RASSF1A gene, again demonstrating the physiological significance of the SETDB1-DNMT3A-HDAC1 complex. To test whether SETDB1 and DNMT3A are associated with each other in the same complex on the RASSF1A promoter, we performed a chromatin immunoprecipitation with antibodies against DNMT3A and then reimmunoprecipitated the cross-linked complexes with antibodies against SETDB1 (sequential ChIP experiment). The reverse experiment was conducted by using SETDB1 antibodies first, followed by DNMT3A antibodies. In both cases, RASSF1A DNA fragments were detected in both primary and secondary immunoprecipitates (Fig. 6C, lanes 3 and 5 and lanes 8 and 10), whereas no PCR signal was observed when nonspecific IgG was substituted for the re-IP antibody (lanes 4 and 9). This demonstrated a simultaneous occupancy of the RASSF1A promoter region by the histone H3-K9 methylase SETDB1 and the DNA methylase DNMT3A.

DISCUSSION

ESET/SETDB1 (ERG-associated protein with a SET domain/SET domain, bifurcated 1), possessing H3-K9 trimethylation activity, was independently identified through interaction with ERG (ets-related gene) and KAP1 (KRAB-associated protein 1) (9, 10). ESET/SETDB1 has been demonstrated to interact with HDAC1/2 (17), mSin3A/3B (17), HP1 (42), and MBD1 (27), whereas its interaction with DNMTs, another important component of the heterochromatin formation and gene silencing machinery, was left untested. Although DNA methyltransferases have been demonstrated to associate with H3-K9 HMT activity (47), which was concluded to be provided by SUV39H1, other recently identified HMTs, such as ESET/SETDB1 have not been studied. Importantly, the physiological and functional significance of the interaction between these two epigenetic layers is not fully understood. Here we report that SETDB1 specifically interacts with DNMT3s but not DNMT1 in vitro and in vivo. DNMT3B3, the DNMT3B splicing variant without methylation activity, also interacted with SETDB1, which suggests involvements of SETDB1 in both DNA methylase activity-dependent and -independent pathways. Structurally, SETDB1 is divided into an N-terminal Tudor domain, central MBD (methylated DNA binding domain), and a large C-terminal bifurcated SET domain. Besides the PHD in DNMT3A and DNMT3B, the N-terminal part of SETDB1 also interacts with the PHD in KAP1 (9). The PHD is thought to mediate protein-protein interactions. Whether SETDB1 could interact with additional PHD-containing proteins and thus regulate their function in distinct pathways through its HMT activity remains to be tested.

Neither SETDB1 nor DNMT3A bind to specific DNA sequences, suggesting that gene-specific de novo methylation of both histone and DNA is controlled by factors that recruit these two enzymes. HP1-mediated recruitment of DNMT3s (24) and MBD1-mediated SETDB1 recruitment (27, 48) have been demonstrated, which supports the histone methylation-dependent DNA methylation model and DNA methylation-dependent histone methylation model, respectively, recently proposed in different contexts (24, 27). In addition, transcription factors, such as c-Myc, have been reported to bring DNMT3A to the p21Cip1 promoter (49), where DNA methylation occurs, whereas through transcriptional corepressor KAP1, SETDB1 regulates KRAB zinc finger protein-mediated gene silencing (9).

In this study, we employed Gal4 binding sites containing artificial promoters and Gal4DBD-fused SETDB1 and DNMT3A to test recruitments of epigenetic modifying enzymes as well as statuses of enzyme-mediated modification. We find that both SETDB1 and DNMT3A are located on the promoter, regardless of which protein fuses to Gal4DBD, and co-expression is required to fully repress promoter activity. Accompanying SETDB1 recruitment to the promoter region, H3-K9 trimethylation in this region was detected, demonstrating the effectiveness of the HMT activity of SETDB1. On the contrary, no promoter DNA methylation has been detected, although Gal4DBD-DNMT3A could directly associate with reporter p6×Gal4-VHL-luciferase. The observed repression activity of DNMT3A on the unmethylated promoter in the tethering assay could therefore be due to endogenous interacting partners, such as HDAC1, whose recruitment was demonstrated by the reporter co-IP assays. Both DNMT3A and ESET/SETDB1 interact with HDAC1 directly (16, 17), whereas our results indicate a direct interaction between DNMT3A and SETDB1, which suggests the formation of a complex that was supported by their simultaneous recruitments to both artificial VHL and endogenous RASSF1A promoters. The fact that each component can bind to the other two should potentially produce a relatively stable complex, and this stability may contribute to gene silencing.

The H3-K9 trimethylation pattern in the p53BP2 promoter
region is maintained by SETDB1 in a replication-coupled, DNA methylation-dependent manner, where MBD1 serves as the adapter protein to deliver SETDB1 to a DNMT1 marked region without requiring direct interaction between SETDB1 and DNMT1 (27). Indeed, our results did not reveal any interaction between SETDB1 and DNMT1, which may suggest that promoters targeted by SETDB1 may perhaps be limited to those controlled by MBD1 instead of all hypermethylated regions. In contrast to SUV39H1 that binds to both DNMT1 and DNMT3A (47), SETDB1 specifically interacts with the de novo DNA methylase DNMT3A. The physiological significance of this specific interaction was evidenced by the co-localization of these two proteins on the methylated p53BP2 and RASSFIA promoter regions. Our data support a self-propagating epigenetic cycle, in which SETDB1 methylates H3-K9 at methylated DNA regions and recruits DNMT3A to reinforce DNA methylation maintenance. Recently, an important involvement of DNA regions and recruits DNMT3A to reinforce DNA methylation maintenance. SETDB1 specifically interacts with the de novo DNA methylase DNMT3A. The physiological significance of this specific interaction was evidenced by the co-localization of these two proteins on the methylated p53BP2 and RASSFIA promoter regions. Our data support a self-propagating epigenetic cycle, in which SETDB1 methylates H3-K9 at methylated DNA regions and recruits DNMT3A to reinforce DNA methylation maintenance. Recently, an important involvement of DNA regions and recruits DNMT3A to reinforce DNA methylation maintenance. SETDB1 specifically interacts with the de novo DNA methylase DNMT3A.

Besides maintaining heritable marks, it is also possible that SETDB1 participates in de novo epigenetic silencing involving both DNA methylation and histone H3-K9 trimethylation. Interestingly, H3-K9 trimethylation is required for DNA cytosine methylation in Neurospora crassa (22). Methylation of the promoter of the tumor suppressor gene RASSFIA has been well documented in many cancers (45, 50); however, mechanisms of this aberrant DNA methylation remain largely unclear. Recently, in proliferating mammary epithelial cells, H3-K9 trimethylation was reported to occur prior to DNA methylation, suggesting a model in which histone methylation triggers de novo DNA methylation of the RASSFIA promoter (46). In ChIP assays conducted in MDA-MB-231 breast cancer cells in which the RASSFIA promoter is hypermethylated (37), trimethylation of H3-K9 was observed at the CpG island of the RASSFIA gene, where SETDB1 occupancy was detected (Fig. 6B). Promoter-bound SETDB1 was shown to trigger H3-K9 trimethylation (Fig. 6); therefore, our results suggest that endogenous SETDB1 is at least partially responsible for the H3-K9 trimethylation of nucleosomes around the RASSFIA promoter. Although the mechanism of how SETDB1 is recruited to the RASSFIA promoter needs further study, MBD1 does not seem to be involved, since in MDA-MB-231 cells, the hypermethylated RASSFIA promoter is specifically bound by MeCP2 instead (51).

We show that SETDB1 is localized to the RASSFIA promoter together with DNMT3A (Fig. 6B). Functionally, these two proteins require each other to fully repress artificial promoter activity. Physically, they colocalize on the endogenous p53BP2 and RASSFIA promoters. Taken together, our results suggest a more detailed scenario regarding RASSFIA promoter methylation. In this process, the SETDB1-HDAC1 complex is first recruited to the unmethylated RASSFIA promoter, perhaps by certain DNA binding transcriptional repressors. Histone tails are then subjected to repressive modifications, and gene transcription is repressed. As SETDB1 and trimethylated H3-K9 accumulate during increased cell passages, DNMT3A is then recruited to the promoter region through its direct interaction with SETDB1 and initiates de novo DNA methylation. Last, as a permanent inactive state of RASSFIA is established, epigenetic modification patterns are stably inherited through the self-reinforcing heterochromatin machinery composed of histone and DNA methyltransferases.

Acknowledgments—We thank Jeanne LeBon and Steven Bates for help with DNA methyltransferase assays and cell culture, respectively.

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