DNA methylation plays an important role in cellular differentiation and development (for a review, see Ref. 1), parental imprinting, X chromosome inactivation, silencing of endogenous retroviruses as well as foreign DNA, and preservation of genome integrity. Changes in the DNA methylation pattern are associated with human cancers, where there is global hypomethylation and promoter-specific hypermethylation (for a review, see Ref. 2). So far, three DNA methyltransferases (DNMTs) have been identified in mammalian cells: DNMT1, also known as the maintenance methylase (3, 4), and DNMT3a and DNMT3b, the two de novo DNA methyltransferases (5).

Purified recombinant DNMT3a and DNMT3b can methylate DNA in vitro without preference for hemimethylated DNA (6–8). The de novo methylation activity of DNMT3a and -3b was also demonstrated in vivo, where ectopic expression leads to methylation of episomal plasmid DNA (9). Inactivation of both genes blocks de novo methylation in embryonic stem cells and early mouse embryos, and these double homozygous embryos die before embryonic day 11.5 (10).

DNMT3a and DNMT3b are highly related proteins that share a PWPP motif (highly conserved proline-tryptophan-proline motif) and an ATRX-like plant homeodomain (PHD) in the N terminus regulatory domain. Disruption of the PWPP motif prevents the association of DNMT3a and -3b with pericentric heterochromatin and abolishes their ability to methylate the major satellite repeats (11). The ATRX-like plant homeodomain in DNMT3a and -3b shares homology with a similar region found in ATRX (12) but lacks the characteristic histidine residue present in typical plant homeodomain fingers (13). The ATRX-like plant homeodomain is responsible for most of the transcriptional repression mediated by DNMT3a and 3b (14) and for interaction with HDAC1 (histone deacetylase-1) and RP58 (a putative transcriptional repressor) (15). Although there is clear evidence for the important role DNA methylation plays in development and cancer, it is still unclear what leads to promoter hypermethylation in cancer and how the enzymatic activity and localization of the DNA methyltransferases are regulated.

NEDD8 (neural precursor cell-expressed developmentally down-regulated 8) or RUB1 (related to ubiquitin 1) is the closest homolog to ubiquitin, with 60% identity and 80% homology (16). NEDD8, similar to ubiquitin and ubiquitin-like proteins, can be covalently attached to other proteins in a process called NEDDylation. NEDDylation is essential for the viability of most model organisms, including Schizosaccharomyces pombe, Cae-norhabditis elegans, Drosophila, Arabidopsis, and mice (17–22). NEDD8 is conjugated to target proteins in order to regulate their activity, through modulation of protein–protein interactions. The most abundant substrates of NEDD8 are a family of scaffold proteins, called cullins. Cullins are essential for the assembly of several multiprotein E3 ubiquitin ligase complexes that are active only when the cullins are modified by NEDD8 (23–26). Immunocytochemical analysis showed that NEDD8-conjugated proteins are highly enriched in the nucleus, suggesting nuclear functions for NEDD8. Recent papers have revealed essential roles for NEDD8 modification of Cul4A in heterochromatin formation in yeast (27) and heterochromatin formation as well as DNA methylation in Neurospora (28). In both cases, deletion of Cul4 disrupts heterochromatin formation, and the mutant strains can be complemented with Cul4 but not with Cul4 that cannot be NEDDylated. The mammalian homolog of Cul4, Cul4A, was shown to be associated with repressed chromatin, and its suppression by small interfering RNA...
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(siRNA) reduced histone 3 Lys9 and Lys27 methylation, which are markers of heterochromatin (29).

Here we describe the association of DNMT3b with NEDDylated proteins. We have found that DNMT3b is associated with endogenous NEDD8-modified proteins at a size between 85 and 90 kDa, the size of NEDDylated cullins, and indeed DNMT3b interacts with CUL1, CUL2, CUL3, CUL4A, and CUL5. We confirmed that DNMT3b interacts with endogenous CUL4A and mapped the interaction to the C terminus of CUL4A. Moreover, DNMT3b interacts preferentially with the NEDDylated form of CUL4A. Expression of DNMT3b increased the recruitment of CUL4A and NEDD8 to chromatin. Chromatin immunoprecipitation assays in cells with knock-out of Dnmt3b showed reduced association of CUL4A and NEDD8 with a repressed promoter. Furthermore, by modulating the NEDDylation pathway, we were able to regulate de novo methylation. These results support a connection between DNA methylation and protein NEDDylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Plasmid DNAs—HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium, and HCT-116 cells were grown in McCoy’s 5A modified medium. Medium was supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HEK 293T cells were transfected by calcium phosphate. For chromatin immunoprecipitation (ChIP) assays, HEK 293T cells were transfected by Lipofectamine 2000 reagent (Invitrogen), and for experiments involving siRNA, cells were transfected with Effectene reagent (Qiagen). HCT-116 cells and Dnmt3b KO (30) cells were kindly provided by Bert Vogelstein. DNMT constructs were described previously (14). NEDD8 was amplified by RT-PCR using cDNA from Akata cells and cloned into p3XFLAG-CMV (E4401, Sigma) or pProEX HTc His6 (Invitrogen). NEDD8 mutations were introduced by PCR mutagenesis. HA-CUL1, -2, -3, and -5 (31), Myc-CUL4A (32), CUL4A-DN (Addgene plasmid 15821) (33), CUL4A Δ N100 (Addgene plasmid 19953) (34), mouse Dnmt3b deletion mutants (35), 5xGAL4TKCAT (36), and pREP8-RTA (37) were described previously.

Immunoprecipitation and GST Affinity Assays—For Immunoprecipitation assays, HEK 293T cells in 10-cm dishes were transfected with 10 μg of total DNA using calcium phosphate precipitation. 48 h after transfection, cells were lysed in 1 ml of radioimmune precipitation buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS plus protease inhibitors (0.5 mM PMSF, 2 μg/ml aprotinin, and 10 μg/ml leupeptin)) and 5 mM β-ethylmalimide (NEM) (Sigma), sonicated for 10 s, and cleared by centrifugation. Extracts were precleared using protein G PLUS-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and immunoprecipitated with anti-FLAG M2-agarose (Sigma) or anti-HA Agarose (Sigma). Beads were washed six times with radioimmune precipitation buffer or with high salt buffer (50 mM Tris, pH 7.9, 1 M NaCl, 0.5 mM EDTA, 2% glyc erol, 0.2% Nonidet P-40), and bound proteins were detected by Western blotting. Immunoprecipitation of endogenous NEDDylated proteins was performed with high salt extraction, high speed centrifugation as described previously (38). The antibodies used for Western blot analysis included mouse anti-HA (Sigma), rabbit anti-FLAG (Sigma), rabbit anti-GAL4 (Upstate), rabbit anti-DNMT3b (Affinity BioReagents), rabbit anti-NEDD8 (ZYMED), rabbit anti-CUL4A (Cell Signaling), mouse anti-Myc (Millipore), and mouse anti-β-actin (Sigma) antibodies. GST affinity (pull-down) assays were performed as described previously (39), with the exception that recombinant GST or GST-DNMT3b fused proteins were incubated with recombinant His6-NEDD8, and the eluted bound proteins were subjected to Western blotting with anti-NEDD8 or anti-DNMT3b antibodies.

DNA Methyltransferase Assays—Assays in transfected HEK 293T cells were performed as described previously (37). For experiments involving siRNA, cells were transfected with siRNA control or siRNA against NAE1/APPBP1 (SMARTpool, DHARMACON Inc.) by Effectene reagent (Qiagen). After 7 h, transfected cells were replated and cultured overnight. The next day, cells were transfected again with siRNAs plus pREP8-RTA and plasmid expressing DNMT3b. At 24 h post-transfection, cells were replated onto 10-cm plates and cultured for another 3 days. DNA was purified by Hirt extraction and digested by HhaI prior to Southern blotting or real-time PCR with primers (sense, AGCGTATGCTTCAGGACCAC; antisense, ACAGATGTGGCATTGCGTA) overlapping the HhaI restriction site in plasmid pREP8-RTA.

Chromatin Immunoprecipitation Assays—ChIP assays with HCT cell lines were performed on HCT 116 or HCT 116 with a deletion of both alleles of Dnmt3b (3b KO). ChIP assays with HEK 293T cells were performed on cells that were transfected with a plasmid that contains 5xGAL4 UAS (5xGAL4TKCAT) with or without GAL4-DNMT3b expression vector. Antibodies against NEDD8 and CUL4A were used to immunoprecipitate endogenously expressed proteins. ChIP assays were performed essentially as described previously (40), except that after proteinase K, the DNA was purified with a PCR purification kit. Ultra Clean PCR clean-up kit, MO BIO Laboratories, Inc.). ChIP DNA was analyzed by real-time PCR (iCycler, Bio-Rad) on products generated with SYBR Green PCR master mix (Applied Biosystems) and primers for PCDH10 promoter (sense, GCTCGGTGTCCTCCTCATTT; antisense, ATGCCTGCGCAGAAGTTT) (41) or 5xGAL4 UAS (sense, CTC-TAGAGTCGAGCTAGAGT; antisense, CCCCTGAAGCTTGCATGCC).

RESULTS

DNMT3b and DNMT3a Associate with NEDD8-modified Proteins—Because the cellular DNMTs can lead to promoter hypermethylation and transcription silencing, their activity and recruitment to different promoters must be tightly regulated. Previously, it was shown that DNMT3a (42, 43), DNMT3b (44), and recently DNMT1 (45) are modified by the ubiquitin-like protein SUMO-1. We investigated whether the DNMTs (DNMT1, DNMT3a, and DNMT3b) are subjected to regulation by other small ubiquitin modifiers and discovered an interaction with NEDD8-modified proteins. Cells were transfected with FLAG-NEDD8 and HA-DNMTs, and cell extracts were immunoprecipitated with anti-FLAG-conjugated beads to immunoprecipitate NEDD8 and NEDD8-associated proteins.
Both DNMT3a and DNMT3b were immunoprecipitated with NEDD8 (Fig. 1, top), whereas DNMT1 was not efficiently immunoprecipitated under these conditions.

The co-immunoprecipitation of DNMT3b with NEDD8 can be explained by either interaction or modification of DNMT3b by NEDD8. To distinguish between these two possibilities, we performed a reciprocal immunoprecipitation. Extracts from cells transfected with HA-DNMT3b were immunoprecipitated with anti-HA-conjugated beads to immunoprecipitate DNMT3b and associated endogenous NEDD8 and NEDD8-conjugated proteins. We observed that DNMT3b co-immunoprecipitated with NEDD8-modified proteins of ~85–90 kDa (Fig. 2, top). These NEDDylated bands are smaller than the size of HA-tagged DNMT3b (~130 kDa), making it unlikely that NEDDylated DNMT3b is being detected. These experiments suggest that DNMT3b associates with NEDD8-modified proteins.

**DNMT3b Preferentially Interacts with Conjugated NEDD8**

If NEDD8 modification regulates the interaction of DNMT3b with different proteins, DNMT3b should preferentially interact with conjugated NEDD8 over unconjugated free NEDD8. We performed immunoprecipitations to examine whether a mutant NEDD8 that cannot be conjugated is able to co-immunoprecipitate DNMT3b. Because the diglycine in NEDD8 (as well as in ubiquitin and ubiquitin-like proteins) is essential for conjugation, we introduced a deletion of the diglycine (NEDD8ΔGG). Although DNMT3b co-immunoprecipitated efficiently with wild type NEDD8, it was not efficiently co-immunoprecipitated with the ΔGG mutant (Fig. 3A, top). This result indicates that DNMT3b preferentially interacts with conjugated NEDD8.

The above results demonstrate that DNMT3b is associated with NEDD8 in mammalian cells. Because proteins are often found in multisubunit protein complexes and are immunoprecipitated with their protein complex, the interaction that we observed could be mediated through additional proteins. In order to test whether DNMT3b and NEDD8 interact directly, we performed a series of pull-down assays. Bacterially expressed and purified recombinant His$_8$-NEDD8 was incubated with GST-DNMT3b or GST that were immobilized on glutathione beads. Bound proteins were eluted and analyzed by Western blotting (Fig. 3B). Recombinant NEDD8 was bound to GST-DNMT3b and not GST alone, suggesting that direct interaction between NEDD8 and DNMT3b can occur.

Protein NEDDylation is a balance between NEDDylation and de-NEDDylation processes. In a **DEN1** (NEDD8 de-NEDDylating enzyme, also known as NEDP1 and SENP8) null mutant in *Drosophila*, many cellular proteins become highly NEDDylated (46), suggesting that many more proteins are NEDDylated in the cell than are currently recognized. In order to push the balance toward protein NEDDylation, we overexpressed FLAG-NEDD8. Cells were co-transfected with HA-DNMT3b and FLAG-NEDD8, and cell extracts were immunoprecipitated with anti-HA-conjugated beads. We observed that DNMT3b co-immunoprecipitated with multiple NEDD8-modified proteins when the blots were probed with anti-FLAG for FLAG-NEDD8 conjugated proteins (Fig. 3C, top). CULLIN-5 (CUL5), a known NEDD8-modified protein, served as a control (Fig. 3D). In contrast to the many NEDDylated proteins associated with DNMT3b, CUL5 immunoprecipitated as a single NEDDylated band (Fig. 3D, top), as expected for a NEDDylated protein. Overexpression of NEDD8 leads to the appearance of the
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FIGURE 3. DNMT3b interacts with NEDD8 in vitro, and preferentially with conjugated NEDD8 in vivo. A, DNMT3b interacts preferentially with conjugated NEDD8. HEK 293T cells were transfected with expression vectors for HA-DNMT3b and FLAG-NEDD8 or a mutant without the C terminus GG (FLAG-NEDD8 Δ GG). Total cell extracts were immunoprecipitated (IP) with anti-FLAG beads and subjected to SDS-PAGE and Western blot analysis (WB). Co-immunoprecipitated DNMT3b (top) and input DNMT3b (middle) were detected with anti-HA antibody. NEDDylation of FLAG-NEDD8 WT and mutant were detected using anti-FLAG antibody (bottom). B, DNMT3b interacts directly with NEDD8 in vitro. Bacterially expressed His₆-NEDD8 was incubated with GST or GST-DNMT3b in the presence of Glutathione beads and bound proteins were analyzed by Western blotting. C, HEK 293T cells were transfected with HA-DNMT3b and FLAG-NEDD8 expression vectors, and total cell extracts were immunoprecipitated with anti-HA-conjugated beads and subjected to SDS-PAGE and Western blot analysis. Immunoprecipitated NEDDylated proteins were detected with anti-FLAG antibody (top). Verification of NEDDylation was shown by anti-NEDD8 antibody after direct immunoprecipitation with anti-FLAG-conjugated beads (middle). The presence of HA-DNMT3b in the immunoprecipitates was detected with anti-HA antibody (bottom). D, as in C, except that HA-CUL5 was transfected instead of HA-DNMT3b. CUL5, a known NEDDylated protein, served as a positive control.

expected higher molecular weight band of CUL5 (Fig. 3D, bottom). The difference in behavior of DNMT3b and CUL5 supports the notion that DNMT3b interacts with NEDD8-conjugated proteins.

__DNMT3b Interacts with NEDDylated Proteins through a Region between Its ATRX-like PHD and DNA Methyltransferase Domains—__To further validate interaction of DNMT3b with NEDDylated proteins, we performed a series of immunoprecipitations with several different DNMT3b deletion mutants. HEK 293T cells were transfected with FLAG-NEDD8 together with GAL4-DNMT3b or the N terminus of DNMT3b (GAL4-DNMT3b) (aa 1–515), and protein extracts were subjected to immunoprecipitation with anti-FLAG-conjugated beads (Fig. 4A). Although GAL4-DNMT3b was co-immunoprecipitated with NEDD8, GAL4-DNMT3b 1–515 was not. This result shows that there is no interaction domain within the N terminus of DNMT3b. In order to narrow down the interaction domain, we next used a series of mouse DNMT3b mutants (Fig. 4B). Full-length (FL) DNMT3b as well as DNMT3b 1–583 and DNMT3b deleted for the ATRX-like PHD domain (Δ433–532) were able to interact with NEDD8 in a co-immunoprecipitation assay, but the mutant DNMT3b 1–433 failed to interact with NEDD8. This experiment shows that mouse DNMT3b has the ability to associate with NEDD8-modified proteins and that a small region between 532 and 583 of mouse DNMT3b is required for interaction. This set of assays maps the region between the ATRX-like PHD domain and the catalytic domain of DNMT3b as the interaction domain for NEDDylated proteins (Fig. 4C).

Because conjugation of NEDD8 is typically on a lysine residue, we mutated the two lysines K540R and K562R present in the aa 532–583 region of DNMT3b. Both mutants retained their ability to associate with NEDD8-modified proteins and that a small region between 532 and 583 of mouse DNMT3b is required for interaction. This set of assays maps the region between the ATRX-like PHD domain and the catalytic domain of DNMT3b as the interaction domain for NEDDylated proteins and is not modified by NEDD8.

__NEDD8 Increases DNMT3b-dependent DNA Methylation—__After confirming the interaction between DNMT3b and NEDD8-conjugated proteins, we sought to test the role of NEDD8-modified proteins in DNA methylation. We performed an in vivo methylation assay (9), using HEK 293T cells transfected with an episomal target plasmid DNA containing a known methylation-regulated herpesvirus promoter (37, 47).

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We co-transfected NEDD8 in combination with DNMT3b and the target plasmid (pREP8-RTA). Five days after transfection, DNA was isolated from the cells and subjected to methylation-sensitive restriction enzyme digestion (HhaI), and the digested DNA was analyzed by Southern blotting (Fig. 5A) or by real-time PCR using primers surrounding the HhaI restriction site (Fig. 5B). Exogenous expression of NEDD8 alone did not lead to any detectable methylation because the assay is dependent on co-expression of de novo methyltransferase in these cells. As expected, expression of DNMT3b resulted in detectable DNA methylation. Interestingly, co-expression of NEDD8 and DNMT3b resulted in a more profound DNA methylation, suggesting that NEDD8-conjugated proteins play a role in DNMT3b-dependent DNA methylation. An in vivo DNA methylation assay was also performed where the first enzyme (E1) in the NEDDylation pathway, NAE1, was suppressed by siRNA. Suppression of NAE1 levels by siRNA was confirmed by reverse transcription followed by real-time PCR (Fig. 5C). Suppression of NAE1 inhibited DNMT3b-dependent DNA methylation (Fig. 5D), as compared with siControl. Interestingly, the level of DNA methylation was inhibited to the same level that NAE1 was suppressed. These experiments suggest that NEDDylation can control DNA methylation; higher levels of NEDDylation enhance DNA methylation, whereas lower levels of NEDDylation attenuate DNA methylation.

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Recent papers outline an essential role for NEDD8 modification of Cul4 in heterochromatin formation in yeast (27) and heterochromatin formation as well as DNA methylation in Neurospora (28). Our immunoprecipitation assays suggest that DNMT3b associates with endogenously NEDDylated proteins of a size between 85 and 90 kDa (Fig. 2, lane 2, top). Cullins are the most abundant NEDD8-conjugated proteins, and they migrate as ~85–90-kDa proteins. Cullins (CUL-1, -2, -3, -4A, -4B, and -5) are scaffold proteins for several multisubunit E3 ubiquitin ligases, and modification with NEDD8 at the C terminus is a prerequisite for their function (23–26). Co-immunoprecipitation experiments were performed to test the ability of DNMT3b to interact with endogenous cullins. Cell extracts
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FIGURE 5. NEDD8 enhances DNMT3b-dependent DNA methylation. A, pREP8-RTA target DNA was transfected into HEK 293T cells with or without NEDD8 or DNMT3b. Plasmid DNA was isolated by Hirt extraction 5 days after transfection and digested with the methylation-sensitive restriction enzyme HhaI, and the DNA was examined by Southern blotting. M, methylation-induced bands; U, unmethylated bands. B, analysis of the HhaI-digested DNA (from A) by real-time PCR using primers surrounding an HhaI restriction site in the RTA promoter. The data are expressed as a ratio of the signal obtained from HhaI-digested undigested DNA. C, cells were transfected with siRNA control or siRNA against NAE1. After 72 h, RNA was isolated, and reverse transcription real-time PCR was performed. The ratio of NAE1 level relative to actin is presented. Error bars, S.D.

from cells transfected with HA-DNMT3b were immunoprecipitated with anti-HA-conjugated beads, and endogenously co-immunoprecipitated proteins were detected with anti-NEDD8, anti-CUL5, and anti-CUL4A antibodies. We found that DNMT3b interacts with endogenous CUL5 and CUL4A as well as with NEDD8 (Fig. 6A).

To examine whether other cullin family members are able to interact with DNMT3b, cells were co-transfected with FLAG-tagged DNMT3b and HA-tagged CUL1, CUL2, CUL3, and CUL5 (Fig. 6B). Cell extracts were immunoprecipitated with anti-FLAG-conjugated beads and blotted with anti-HA antibodies. CUL1, CUL2, CUL3, and CUL5 were detected in the immunoprecipitates. These data demonstrate that DNMT3b has the ability to interact with multiple cullins, the most abundant targets for NEDDylation in the cell.

DNMT3b Preferentially Interacts with NEDDylated CUL4A—In order to check the role of NEDD8 modification on DNMT3b and CUL4A interaction, cell lysis and immunoprecipitation procedures were performed with or without NEM (N-ethylmaleimide). NEM is a cysteine protease inhibitor that inhibits deconjugation of ubiquitin and ubiquitin-like proteins and therefore is used routinely to maintain NEDD8 modification.

Cells were transfected with HA-DNMT3b, and 2 days after transfection, the cells were collected into two tubes; in one tube, protein extract was prepared and immunoprecipitated with NEM, and in the second tube, immunoprecipitation was done without NEM. Anti-CUL4A was used to detect endogenous CUL4A that was co-immunoprecipitated with DNMT3b. Two protein bands of CUL4A were detected in the input in the presence of NEM, but the slow migrating band corresponding to NEDDylated CUL4A was markedly reduced without NEM (Fig. 7A, top, lanes 3 and 4). Interestingly, DNMT3b was associated specifically with NEDDylated CUL4A and only in the presence of NEM (Fig. 7A, top, lanes 1 and 2). The immunoprecipitation was also blotted with anti-NEDD8 antibody (Fig. 7A, middle) to confirm that the modification is NEDDylation. This result indicates that DNMT3b interacts preferentially with the NEDDylated form of endogenous CUL4A.

Next, we mapped the interaction domain on CUL4A. Cells were co-transfected with DNMT3b and Myc-tagged CUL4A or CUL4A ΔN100 (N terminus aa 1–100 deleted) or CUL4A-DN (deletion of aa 440–759), and immunoprecipitations were performed with anti-FLAG-conjugated (Fig. 7, B and C) or anti-HA-conjugated (Fig. 7D) beads to immunoprecipitate DNMT3b. DNMT3b co-immunoprecipitated with CUL4A WT (Fig. 7, B and D) and CUL4A ΔN100 (Fig. 7C), but not with CUL4A-DN (Fig. 7D). In Fig. 6A, we found that DNMT3b interacts with endogenous CUL4A, and here (Fig. 7B) we confirm the interaction with a transfected CUL4A. Furthermore, this immunoprecipitation assay maps the C terminus of CUL4A as the interaction domain with DNMT3b.

An Inactive CUL4A Mutant Impairs DNMT3b-dependent DNA Methylation—Deletion of aa 1–100 from the N terminus of CUL4A abolishes its ability to bind to DDB1 and to ubiquitinate substrates such as CDT1 (48). DNMT3b interacts with this mutant (Fig. 7C); therefore, this mutant may act as a dominant negative. We used this mutant to examine the importance of CUL4A in DNMT3b DNA methylation in vivo. Cells were transfected with DNMT3b and CUL4A ΔN100 together with the target plasmid, and methylation of the target DNA was analyzed by HhaI digestion followed by real-time PCR (Fig. 7E). The expression of CUL4A ΔN100 indeed impaired DNMT3b-dependent DNA methylation. This suggests a role for active CUL4A in DNA methylation by DNMT3b.
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FIGURE 6. DNMT3b interacts with cullins. A, DNMT3b interacts with endogenous CUL4A and CUL5. Extracts of HA-DNMT3b-transfected HEK 293T cells were immunoprecipitated (IP) with anti-HA-conjugated beads and subjected to SDS-PAGE and Western blot analysis (WB). Specific antibodies against NEDDylated proteins (top panel), CUL5 (upper middle panel), and CUL4A (lower middle panel) were used to detect endogenous proteins in the inputs (lanes 3 and 4) and in the immunoprecipitates (lanes 1 and 2). The presence of HA-DNMT3b in the immunoprecipitates was detected with anti-DNMT3b antibody (bottom panel). B, DNMT3b interacts with CUL1, CUL2, CUL3, and CUL5. FLAG-DNMT3b and HA-tagged CUL1, CUL2, CUL3, and CUL5 were co-transfected into HEK 293T cells, and cell extracts were immunoprecipitated with anti-FLAG-conjugated beads. Cullins were detected in the immunoprecipitates (panel) and the input (middle) using anti-HA antibody. Expression of FLAG-DNMT3b was detected with anti-FLAG antibody (bottom).

Recruitment of CUL4A and NEDD8 to Chromatin by DNMT3b—One possible outcome of the interaction between DNMT3b and CUL4A is that DNMT3b has a role in the recruitment of CUL4A to repressed promoters. In order to test a direct role for DNMT3b in the recruitment of NEDD8 and CUL4A to chromatin, we performed ChIP assays on cells that were transfected with DNMT3b fused to the GAL4 DNA binding domain together with a plasmid that contains GAL4 consensus binding sites. Endogenous NEDD8 and CUL4A were immunoprecipitated, and their association with the plasmid DNA with or without GAL4-DNMT3b was analyzed by real-time PCR (Fig. 8, A–C). The association of both NEDD8 and CUL4A with the plasmid DNA was increased in the presence of GAL4-DNMT3b. These results indicate a specific role for DNMT3b in the recruitment of CUL4A and NEDD8 to chromatin.

Deletion of Dnmt3b Reduced the Association of CUL4A and NEDD8 at a Repressed Promoter—To examine whether CUL4A recruitment to a repressed promoter in cancer cells is also dependent on DNMT3b, we performed the ChIP assay in two cell lines, HCT 116 (human colon carcinoma) or HCT 116 with a knock-out of Dnmt3b (3b KO). We found reduced association of both NEDD8 and CUL4A to a known repressed promoter (PCDH10) in the Dnmt3b−/− cells as compared with the parental cells (Fig. 8D). These results indicate that DNMT3b has a role in the recruitment of CUL4A and NEDD8 to a repressed promoter in cancer cells.

DISCUSSION

Here we describe the interaction of the de novo DNA methyltransferase DNMT3b with NEDD8-modified proteins. DNMT3b immunoprecipitates two major bands of endogenously NEDDylated proteins at the size of NEDDylated cullins, and indeed DNMT3b was found to interact with CUL1, CUL2, CUL3, CUL4A, and CUL5. Protein NEDDylation is a balance between NEDDylation and de-NEDDylation processes. When this balance is disrupted, as in the Dnmt3b (NEDD8 de-NEDDylating enzyme) null mutant in Drosophila, many cellular proteins become highly NEDDylated (46). We found that many NEDDylated proteins associated with DNMT3b when the balance toward NEDDylation was biased by NEDD8 ectopic expression. Although DNMT3b can interact directly with NEDD8 at a repressed promoter in cancer cells.

Cullins are the most abundant NEDD8-conjugated proteins in the cell. Cullins are scaffolds for the assembly of multisubunit ubiquitin E3s that ubiquitinate numerous proteins. All cullins are NEDDylated on a conserved lysine in their C terminus domain, and modification by NEDD8 is necessary for cullin activity. Recently, several reports in yeast, Neurospora, and mammalian cells have suggested an essential role for CUL4A and its NEDDylation dependent activity in heterochromatin formation (27, 29). In S. pombe, Cull4 interacts with the histone methyltransferase Clr4 that is responsible for histone 3 lysine 9 methylation across all heterochromatin domains (49). Mutations in Cull4 result in defective localization of Clr4 and a loss of silencing at heterochromatin loci. The heterochromatin defects in Cull4 mutants could not be rescued by expression of a Cull4 protein lacking NEDD8 modification, suggesting a role for a NEDDylated Cull4 in histone 3 lysine 9 methylation and heterochromatin formation (27). Recently, it was shown that Cull4 is essential for DNA methylation in Neurospora (28). Knock-out of Cull4 resulted in a loss of DNA methylation, and the mutant strains were complemented by Cull4 but not by Cull4 that could not be NEDDylated. In mammalian cells, CUL4-DDB1 binds the polycomb group protein EED, a WD40 repeat...
subunit of EZH2 histone methyltransferase complex (29). The EED-EZH2-SUZ12 methyltransferase directs histone H3 methylation at Lys9 and Lys27, leading to X chromosome inactivation, genomic imprinting, heterochromatin formation, and gene silencing. CUL4-DDB1 associates specifically with trimethylated histone 3 Lys9 and histone 3 Lys27 mononucleosomes, and inactivation of CUL4 or DDB1 significantly abolishes trimethylation at histone 3 Lys9 and Lys27 (29).

The results presented here suggest that CUL4A interacts with DNA methyltransferase DNMT3b. ChIP assays revealed that expression of DNMT3b led to enhanced association of CUL4A and NEDDylated proteins that were co-immunoprecipitated with DNMT3b, respectively. WB, Western blot. B, HEK 293T cells were transfected with HA-DNMT3b, and 2 days after transfection, cells were collected into two tubes; in one tube protein extract was prepared and immunoprecipitated (IP) with NEM, and in the second immunoprecipitation was done without NEM. Anti-CUL4A and Anti-NEDD8 antibodies were used to detect endogenous CUL4A and NEDDylated proteins that were co-immunoprecipitated with DNMT3b, respectively. WB, Western blot. C, Myc-CUL4A and FLAG-DNMT3b were detected in the immunoprecipitates (top) and the input (middle) using anti-Myc antibody. The presence of DNMT3b in the immunoprecipitation was detected with anti-FLAG antibody (bottom), as in B but with transfected Myc-CUL4A Δ N100 (deletion of aa 1–100). D, HEK 293T cells were transfected with HA-DNMT3b and CUL4A or CUL4A-DN (aa 1–440, deletion of the C terminus), and immunoprecipitations were performed with anti-HA-conjugated beads. CUL4A and CUL4A-DN were detected in the immunoprecipitates (top) and the input (bottom) using anti-CUL4A antibody. E, an inactive CUL4A mutant attenuates DNMT3b-dependent DNA methylation. pREP8-RTA target DNA was transfected into HEK 293T cells with or without CUL4A Δ N100 or DNMT3b. Methylation of the plasmid DNA was analyzed as in Fig. 5B.

Previously, it has been shown that DNMT1 interacts with an E3 ubiquitin ligase, UHRF1 (ubiquitin-like, containing plant homeodomain and RING finger domains 1), also known as NP95 in mice and ICBP90 in humans (51, 52). This ubiquitin ligase binds histones and ubiquitinates histone 3 (53) and, through its binding to hemimethylated DNA, is essential for the recruitment of DNMT1 to chromatin during replication and to maintain DNA methylation (51, 52). Here we describe an interaction between CUL4A and DNMT3b. The CUL4A ubiquitin ligase complex, consisting of CUL4A-CUL4B-DDB1-DDB2-ROC1, is responsible for H3 and H4 ubiquitination (54). This complex is also responsible for the enhanced ubiquitination of...
H3 and H4 induced by UV irradiation. The fact that DNMT1 and now DNMT3b interact with histone ubiquitin ligase complexes may be relevant to the cellular DNA damage response. It has been shown that both SIRT1 and DNMT3b are recruited to the chromatin after DNA damage (55). Because the CUL4A complex is recruited to DNA damage through DDB1 (56), our work raises the possibility that after DNA damage, the recruitment of both SIRT1 and DNMT3b is through a NEDDylated CUL4A complex. Determination of whether histone ubiquitination also has a role in DNMT-mediated transcription repression and DNA methylation awaits further study.

In cancer cells, many promoters become aberrantly methylated through the activity of the de novo DNA methyltransferases DNMT3a and DNMT3b and acquire repressive chromatin marks. Because DNMT3a and DNMT3b have the ability to de novo methylate DNA, their activity and recruitment to different promoters must be tightly regulated. The DNMT3L protein is essential for de novo DNA methylation at maternal genomic imprints (57) and regulates the activity of DNMT3a (58) and DNMT3b (59) through chromatin recruitment (60).

Suggested that higher levels of NEDD8 conjugation are necessary for the high proliferative property of many, if not all, carcinoma cell lines (67). A recent report found that DNMT3b overexpression correlates with higher DNA methyltransferase activity in breast cancer cell lines (68). It will be interesting to test a possible link between DNA methylation and NEDD8 expression in different tumor types to see if both are concurrently up-regulated.

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