Truncated DNMT3B Isoform DNMT3B7 Suppresses Growth, Induces Differentiation, and Alters DNA Methylation in Human Neuroblastoma

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Abstract

Epigenetic changes in pediatric neuroblastoma may contribute to the aggressive pathophysiology of this disease, but little is known about the basis for such changes. In this study, we examined a role for the DNA methyltransferase DNMT3B, in particular, the truncated isoform DNMT3B7, which is generated frequently in cancer. To investigate if aberrant DNMT3B transcripts alter DNA methylation, gene expression, and phenotypic character in neuroblastoma, we measured DNMT3B expression in primary tumors. Higher levels of DNMT3B7 were detected in differentiated ganglioneuroblastomas compared to undifferentiated neuroblastomas, suggesting that expression of DNMT3B7 may induce a less aggressive clinical phenotype. To test this hypothesis, we investigated the effects of enforced DNMT3B7 expression in neuroblastoma cells, finding a significant inhibition of cell proliferation in vitro and angiogenesis and tumor growth in vivo. DNMT3B7-positive cells had higher levels of total genomic methylation and a dramatic decrease in expression of the FOS and JUN family members that comprise AP1 transcription factors. Consistent with an established antagonistic relationship between AP1 expression and retinoic acid receptor activity, increased differentiation was seen in the DNMT3B7-expressing neuroblastoma cells following treatment with all-trans retinoic acid (ATRA) compared to controls. Our results indicate that DNMT3B7 modifies the epigenome in neuroblastoma cells to induce changes in gene expression, inhibit tumor growth, and increase sensitivity to ATRA. Cancer Res; 72(18); 4714–23. ©2012 AACR.

Introduction

In adult cancers, epigenetic changes and aberrant splicing of DNMT3B are observed frequently (1, 2). Pediatric neuroblastoma is characterized by a range of clinical behaviors, and genetic and epigenetic aberrations contribute to pathogenesis (3–5). The Cohn laboratory and others have shown that hypermethylation and silencing of genes involved in the regulation of tumor growth, cell cycle, apoptosis, and DNA repair are associated with aggressive growth and poor outcome in patients (6–8). Further, poor prognosis can be predicted by a CpG island methylator phenotype (9). Preclinical studies have demonstrated that neuroblastoma growth can be inhibited by drugs that disrupt DNA methylation (10, 11). Retinoic acid, a differentiation-inducing agent that has been shown to improve clinical outcome (12), can reverse the methylation status of hundreds of gene promoters (13), possibly due to decreased levels of the DNA methyltransferase (DNMT) enzymes (14).

Three DNMT enzymes regulate DNA methylation in eukaryotic cells (15–17), with widespread aberrant DNMT3B transcription in cancer cells, some encoding truncated proteins lacking the catalytic domain (1, 2, 18–20). Forced expression of DNMT3B7 within 293 cells led to altered DNA methylation levels and corresponding gene expression changes, indicating that DNMT3B7 expression could alter DNA methylation levels and gene expression (18). DNMT3B7 transgenic mice exhibit altered embryonic development (21).

Despite advances in genome-wide profiling that demonstrate strong correlations between genetic aberrations and clinical phenotype, we know much less about the clinical significance of epigenetic changes in neuroblastoma. Given that aberrant DNMT3B isoforms are common in cancer cells,
we hypothesized that aberrant DNMT3B transcripts found in neuroblastoma tumors could alter DNA methylation, gene expression, and tumor phenotype.

Materials and Methods

Patients and tumor specimens
Two ganglioneuroblastoma, three ganglioneuroma, and fourteen primary neuroblastoma tumors were obtained from Children's Memorial Hospital in Chicago under an IRB approved protocol. Twelve RNA samples derived from one ganglioneuroblastoma and eleven neuroblastomas were obtained from the Children's Oncology Group (COG) Neuroblastoma Tumor Bank. The laboratory studies were approved by The University of Chicago Institutional Review Board.

Cell culture, cell identification, and nucleic acid isolation
Cell line identities were verified by short tandem repeat profiling using the AmpF/STR Identifier PCR Amplification Kit (Applied Biosystems). RNA and DNA isolation was performed using TRIzol (Invitrogen) and Puregene Core Kit (Qiagen), respectively.

The MSCV-I-GFP plasmid backbone was engineered to confer constitutive DNMT3B7 expression using a 5’ EcoRI site, C-terminal His-tag epitope, and 3’ XhoI site using primers listed in Supplementary Table S1. Retroviral constructs and packaging plasmid psi-Eco were used to produce retroviral supernatants by 293T cell cotransfection using Fugene6 Transfection Reagent (Roche). The inducible DNMT3B7 construct was generated using oligonucleotides listed in Supplementary Table S1 to ligate into the pUTretX-Tight-Pur response vector (pRXTPL, Clontech Laboratories). The Tet-Off System (Clontech) was used to produce inducible LA1-55n cell lines. In the presence of doxycycline, there is no induction of gene expression in either the control or DNMT3B7-containing cell line. In the absence of doxycycline, the DNMT3B7-containing cells express both GFP and DNMT3B7, and the vector control cells express only GFP. We have compared the vector cells expressing GFP to the experimental cells expressing GFP and DNMT3B7 to control for any effects of GFP expression.

All plasmid inserts were sequenced to ensure proper correct sequence.

Neuroblastoma xenograft studies
Four- to six-week-old female homozygous athymic nude mice (Harlan) were inoculated subcutaneously into the right flank with DNMT3B7-expressing cells (control) and measured twice weekly (see Supplementary Materials and Methods for details). Tumor volumes were calculated using the following formula: tumor volume = (length x width²)/2. Mice bearing SMS-KCNR (constitutive expression) xenografts were sacrificed after 28 days. Mice bearing LA1-55n (inducible expression) xenografts were divided randomly into two groups when palpable tumors developed; control mice continued receiving doxycycline-containing water to block DNMT3B7 expression, and animals in the experimental group were given normal drinking water to induce DNMT3B7 expression. Mice in both groups were sacrificed after 35 days.

Immunohistochemical analysis
Immunohistochemistry was performed for CD-31 (1:100, M-20, Santa Cruz Biotechnology) to determine the mean vascular density, and Ki-67 (1:200, MIB-1, DakoCytomation) to assess proliferation rate (10).

Quantification of apoptosis
In situ detection of apoptosis was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics Corp.; ref. 22).

Liquid chromatography/mass spectroscopy
Total cytosine methylation was performed by liquid chromatography/mass spectroscopy (LC/MS), as described previously (21).

Sodium bisulfite treatment, PCR amplification, and protein expression analysis
Genomic DNA was treated with sodium bisulfite (23), and PCR amplifications were performed using the primers listed in Supplementary Table S1 using ZymoTaq (ZymoResearch) at the indicated temperatures. Reverse transcription was performed using Superscript III (Invitrogen), and PCR amplifications were performed using Platinum Taq (Invitrogen) using primers listed in Supplementary Table S1. Whole cell extracts were made by lysing cells in 75 mmol/L NaCl, 25 mmol/L Tris-Cl, and 1:100 protease inhibitors (CalBiochem). DNMT3B was detected with T-16 antibody (1:500, sc-10236, Santa Cruz Biotechnology), and TOP1 was used as a loading control (1:400, ab3825, AbCam).

RNA-Seq
Total RNAs were isolated using Trizol Reagent (Invitrogen). RNA integrity was validated using the Agilent BioAnalyzer, and all samples had RNA integrity number > 9. Libraries were generated following the Illumina protocol for Preparing Samples for Sequencing of mRNA. PCR amplified cDNA libraries were quantified on an Agilent 2100 Bioanalyzer. Single-end sequencing was performed for 36 cycles using Single Read Cluster Generation Kit V4 (Cat# GD-103-4001) and Sequencing Kits (Cat# FC-104-4002). Sequence reads from the RNA-sequencing (RNA-Seq) were aligned to genomic sequence (Human Feb. 2009 assembly, GRCh37/hg19). RNA-Seq data have been submitted to the GEO database, record number GSE36350.

Statistical analyses
Statistical analysis was performed using a 2-tailed Student’s t test or Fisher’ Exact Test. A P value ≤ 0.05 was considered statistically significant. For the RNA-Seq data, each DNMT3B7-expressing cell line was compared to the vector control and Bonferroni correction was used to correct for multiple testing using n = 18,674, the total number of genes that had at least one read in all 3-cell lines. Analysis of ATRA treatment of DNMT3B7-expressing or control cells was done using the mixed effect restricted maximum likelihood analysis model.
Results

Aberrant DNMT3B transcripts are expressed in primary neuroblastoma tumors

To investigate significance of aberrant DNMT3B transcripts in neuroblastoma, reverse transcriptase-PCR (RT-PCR) analysis was performed on a hypothesis-generating cohort of three ganglioneuroblastoma, three ganglioneuromas, and 25 primary neuroblastoma samples using primers that amplified all but 2 of the known alternative and aberrant DNMT3B isoforms (Fig. 1A, Supplementary Fig. S1A, Table 1). Additional DNMT3B transcripts were detected by Southern blot using a DNMT3B probe that hybridizes to all of the known aberrant isoforms amplified in the RT-PCR (Supplementary Fig. S1B and C). We stratified the patients into two risk groups: high risk and nonhigh risk. Nonhigh-risk patients included those with stages 1 and 2 disease, infants with stages 4 and 4S tumors, and patients with stage 3 tumors that lacked MYCN amplification. Similar to the criteria used by the COG, patients with stage 3 MYCN-amplified tumors and children older than 1 year of age with stage 4 disease were considered high risk (4). We found the expression of four or more aberrant DNMT3B isoforms correlated with higher risk groups (P = 0.03). The identity of DNMT3B7 was confirmed by sequencing in four primary tumors (data not shown), and expression of DNMT3B7 was quantified in the primary tumors by real-time RT-PCR (Supplementary Fig. S2A). We obtained ten more ganglioneuroblastoma RNAs from the COG. However, the integrity of the RNA was too low to determine the levels of DNMT3B7 isoforms (data not shown). We therefore hypothesized that high levels of DNMT3B7 may contribute to the more benign clinical behavior of the most differentiated tumors.

Neuroblastoma cell lines showed expression patterns of DNMT3B transcripts similar to that of the primary tumors by RT-PCR (Fig. 1B). Tumorigenic N-type cells (24) had an increased number of DNMT3B transcripts and lacked DNMT3B7. By Western blot, we found the N-type cells have only full-length DNMT3B, whereas nontumorigenic S-type cells had both full-length DNMT3B and the truncated DNMT3B7 (Fig. 1C).

Forced expression of DNMT3B7 in neuroblastoma cells inhibits growth

Because we detected high levels of DNMT3B7 in differentiated tumors associated with more favorable outcomes, we hypothesized that DNMT3B7 could modify neuroblastoma phenotype. We introduced DNMT3B7 into an N-type neuroblastoma cell line (LA1-55n) using a Tet-off inducible system. Repression of cell growth in the DNMT3B7-expressing cell lines was seen after 10 days of induction (P < 0.001; Fig. 2A). The expression of DNMT3B7 was evaluated by Western blot analysis at each time point (Fig. 2B). DNMT3B7 expression was low during the first week and increased as the growth of the cells decreased. The level of DNMT3B7-expression in the induced cells was two-fold higher than what was found in ganglioneuroblastoma tumors (Supplementary Fig. S2B). Similar results were obtained using constitutive expression of DNMT3B7 in another N-type neuroblastoma cell line, SMS-KCNR (Supplementary Fig. S3).

Figure 1. Aberrant DNMT3B expression and global DNA methylation in primary neuroblastoma tumors. A, RT-PCR examination of aberrant DNMT3B transcripts expressed in neuroblastoma (NB) and ganglioneuroblastoma (GNB) patient samples were normalized to the GAPDH housekeeping gene, and DNMT3B was amplified from exons 6 and 11 to identify the wild-type and aberrant DNMT3B transcripts specifically. DNA sizing is shown on the left and DNMT3B isoforms are shown on the right (ΔDNMT3B). B, RT-PCR examination of aberrant DNMT3B transcripts expressed in neuroblastoma cell lines, normalized to GAPDH housekeeping gene, and DNMT3B was amplified from exons 6 and 11 to specifically identify the wild-type and aberrant DNMT3B transcripts. DNA sizing is shown on the left, and DNMT3B isoforms are shown on the right (ΔDNMT3B). C, identification of truncated DNMT3B proteins in S-type neuroblastoma cell lines by Western blotting. The positions of the molecular weight markers are given on the left. The positions of full-length DNMT3B and truncated DNMT3B7 are indicated on the right. GAPDH was used as a loading control.

Both the inducible DNMT3B7-LA1-55n cells and constitutively-expressing DNMT3B7-SMS-KCNR cells were used to test the effects of DNMT3B7 expression on tumor growth in murine xenograft models using LA1-55n cells (Fig. 3A) and SMS-KCNR...
cells (Fig. 3C). In both models, expression of *DNMT3B* inhibited tumor growth (Fig. 3A and C). Tumor vascularity, cell proliferation, and apoptosis were evaluated in both the LA1-55n (Fig. 3B) and SMS-KCNR (Fig. 3D) xenografts with and without forced *DNMT3B* expression. Histologic sections were stained with CD31 and showed fewer endothelial cells in the *DNMT3B*-expressing tumors (Fig. 3B, iv and D, iv) compared to control tumors (Fig. 3B, i and D, i). Significantly lower mean vascular density (35.2 ± 8.3 vs 72.2 ± 9.5; *P* < 0.001) was detected in xenografts with expression of *DNMT3B* (Supplementary Fig. S4A). In addition, blood vessels in the *DNMT3B*-positive tumors were thin-walled and structurally more normal (Fig. 3B, ii and D, ii) than in the control tumors (Fig. 3B, i and D, i).

Cell proliferation in the *DNMT3B*-positive xenografts was evaluated by Ki-67 expression, a nuclear protein that is preferentially expressed during active phases of the cell cycle. Ki-67 negative cells, representing quiescent G0 phase cells, are more abundant in the *DNMT3B*-expressing tumors (Fig. 3B, vi and D, vi) than in control tumors (Fig. 3B, v and D, v). Significantly higher numbers of neuroblasts in G0 were detected in SMS-KCNR tumors with *DNMT3B* expression, compared to control tumors (39.2 ± 3.5 vs. 14.4 ± 1.9, respectively; *P* < 0.001; Supplementary Fig. S4B). TUNEL assays demonstrated that 2.5-fold more apoptotic cells were present in the *DNMT3B*-positive xenografts compared to controls (*P* < 0.01; Supplementary Fig. S4C). Overall, these results show that expression of *DNMT3B* in two neuroblastoma xenograft mouse models leads to tumors with a less aggressive phenotype.

### Cells with *DNMT3B* expression have higher levels of global DNA methylation

We have shown previously that *DNMT3B* expression in an *Eμ-Myc* transgenic mouse model resulted in an increase in global DNA methylation (21). To determine if the expression of *DNMT3B* in the LA1-55n cells also increases DNA methylation, we measured global DNA methylation levels. As shown in Fig. 4A, significantly higher levels of DNA methylation were detected in the LA1-55n cells expressing *DNMT3B* as compared to vector control cells. We found the Satellite 2 repetitive elements had more DNA methylation following *DNMT3B*-expression in the LA1-55n cells (Supplementary Fig S5). The increase in DNA methylation was also seen in both *DNMT3B*-expressing xenografts. A statistically significant increase in total 5-methylcytosine levels was seen in the *DNMT3B*-expressing xenografts compared to controls (*P* < 0.01, Fig. 4B), indicating that the pattern of DNA methylation is modified in the presence of the truncated *DNMT3B*. Because we identified high levels of *DNMT3B* in ganglioneuroblastomas, we hypothesized that this would correlate with global DNA methylation levels. We found no difference between low, intermediate, and high-risk groups. However, there was a statistically significant increase in

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**Table 1.** *DNMT3B* transcripts expressed in primary ganglioneuroma, ganglioneuroblastoma, and neuroblastoma tumors

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Figure 2. Establishment of neuroblastoma cells overexpressed *DNMT3B*. A, LA1-55n cells were transduced with Tet-controlled transactivator and subsequently with either a Tet-off inducible *DNMT3B* or control vector. One vector clone and two *DNMT3B* clones were grown in the absence of doxycycline for 3 weeks to induce expression. The cells were resuspended and counted every 3 to 4 days and plated at the same density. B, whole cell extracts were made from the vector control and two *DNMT3B*-expressing cell lines and subjected to Western blot analysis using an anti-DNMT3B antibody. Topoisomerase 1 was used as a loading control.
global DNA methylation in the three ganglioneuroblastoma tumors (28, 65-1 and 2235), two of which have the highest level of DNMT3B7 expression seen in any primary tumor (Fig. 4C).

**DNMT3B7 expression induces changes in gene expression and DNA methylation**

To investigate the effects of DNMT3B7 on gene expression, we used RNA-Seq to compare the gene expression profiles of two independent DNMT3B7-expressing LA1-55n cells to vector control cells after 21 days of induction. After eliminating the genes with fewer than 150 reads per cell and those that had less than a three-fold change in either of the two DNMT3B7-expressing cell lines compared to the vector control, there were 144 genes that had at least an average five-fold change in the two DNMT3B7-expressing cell lines compared to the vector control cells (Supplementary Table S2). RNA-Seq confirmed the expression of DNMT3B7 only in the transduced cells (Supplementary Fig. S6). Similar to the expression of DNMT3B7 in Eμ-Myc transgenic mice (21), we found the expression of DNMT1, DNMT3A and endogenous DNMT3B was unchanged in the induced cells according to RNA-Seq, and real-time RT-PCR identified subtle increases in DNMT3A and DNMT3B (Supplementary Fig S7A to C). Among 117 genes with increased expression in the two DNMT3B7-expressing lines, 86 (73.5%) genes contained a CpG island, and 22 of the 27 (81.5%) of the genes with decreased expression contained a CpG island. Interestingly, eight of the 27 (29.6%) genes with decreased expression are found on chromosome 19 (P = 0.0068), and seven (25.9%) are on chromosome 19p (P < 0.0001). Copy number loss of chromosome 19 has been reported in 12.5% of neuroblastoma tumors (25, 26). No other chromosomal clustering of genes altered by DNMT3B7-expression was detected.

To identify genes that could contribute to the suppression of neuroblastoma cell growth in the presence of DNMT3B7, we focused on genes with the greatest fold changes by RNA-Seq that had CpG islands: ANKR12 (22-fold), ASPN (15-fold), and EEA1 (14-fold; increased expression); and FOS (46-fold), FOSB (21-fold), EGR1 (18-fold), and FOS (11-fold; decreased expression). In addition, we performed a GeneGo pathway analysis of all of the genes with at least a five-fold average change in expression with at least 150 reads per cell (Supplementary Table S2), which identified an additional five genes of interest: three genes that encode members of the AP1 complex that were underexpressed in the DNMT3B7-expressing cell lines FOSB (45-fold) and FOS (11-fold), both identified above, and JUNB (6-fold); genes important in neuronal function and tumorigenesis: KIF20B (11-fold), ROCK1 (6-fold), APC (5-fold), and RXRB (6-fold), encoding a retinoic acid signaling coregulator that heterodimerizes with retinoic acid receptors to bind to target gene promoters at the retinoic acid response element, which is important for retinoic acid treatment of children with high-risk neuroblastoma (12). In total, we performed quantitative real-time RT-PCR of all twelve genes of interest (Fig. 5A and B).
DNMT3B7 Suppresses Neuroblastoma Growth

To test if the differential expression of genes in the DNMT3B7-expressing LA1-55n cells was the result of an alteration in their DNA methylation patterns, we performed bisulfite sequencing of eight genes that had been validated by real-time RT-PCR. Six of these genes showed no detectable DNA methylation across the transcriptional start site. *RXRB*, a gene important in retinoic acid signaling, showed a decrease expression of two-fold in DNMT3B7-expressing cells by real-time RT-PCR, and the CpG island surrounding the transcriptional start site was hypermethylated in the presence of DNMT3B7 (Fig. 5C). Similarly, *EEA1* expression was nine-fold higher by real-time RT-PCR, and the CpG island upstream of the transcriptional start site became completely hypomethylated in DNMT3B7-expressing cells (Fig. 5D). The changes in DNA methylation were validated by LC/MS (Supplementary Fig. S8). These data show that expression of DNMT3B7 in neuroblastoma cells leads changes in gene expression, some of which are correlated to altered CpG island methylation.

The effects of ATRA and DNMT3B7 expression on growth inhibition are additive

RNA-Seq of the DNMT3B7-expressing LA1-55n cells revealed decreased expression of genes encoding components of the AP1 complex, *FOSB* (45-fold), *FOS* (11-fold), and *JUNB* (6-fold). In addition to acting as a transcription factor, AP1 can also antagonize the activity of retinoic acid receptors (27). We therefore hypothesized that, with reduced levels of AP1, retinoic acid receptors would be more active and could drive DNMT3B7-expressing cells toward differentiation. To test if retinoic acid treatment could augment the ability of DNMT3B7 to induce differentiation of neuroblastoma cells, we induced DNMT3B7 expression in the LA1-55n cells for 2 weeks to maximize DNMT3B7 expression (Fig. 2B). After 2 weeks of induction, both DNMT3B7-expressing cells and control cells were treated either with all-trans retinoic acid (ATRA) or vehicle for 7 days. We found that the effects of ATRA treatment and DNMT3B7 expression on growth inhibition were additive (Fig. 6A), not synergistic.

To test if DNMT3B7-expressing cells were more differentiated than control cells, we studied the expression of 22 known neuroblastoma differentiation markers (28–33) and found that 18 of them had expression changes that correlated with a more differentiated phenotype (Supplementary Table S3). We validated the two genes with the greatest fold changes: *GFRA1* and *DLK1*. *GFRA1* encodes GDNF family receptor alpha 1 and has been shown to enhance differentiation in response to GDNF (34). *GFRA1* expression was two-fold higher in two independent DNMT3B7-expressing LA1-55n cell lines, compared to vector control (Fig. 6B left, dark gray bars). After ATRA treatment, *GFRA1* expression was further increased in all cell lines (Fig. 6B left, light gray bars), and this increase was greater in the DNMT3B7-expressing cells. *DLK1* encodes delta-like 1 homolog (*Drosophila*) and has been identified as a stem cell gene that negatively regulates differentiation (35). The expression of *DLK1* in DNMT3B7-expressing cells was decreased up to two-fold in two independent DNMT3B7-expressing LA1-55n cell lines (Fig. 6B right, dark gray bars). After ATRA treatment, *DLK1* expression was further decreased in all cell lines (Fig. 6B left, light gray bars), and the expression in the DNMT3B7-expressing cells was significantly lower than in the vector control cells. Taken together, these results suggest a model in which the expression of DNMT3B7 induces neuroblastoma cells to differentiate, which can be promoted further by additional treatment by ATRA.

Discussion

DNMT3B catalyzes de novo methylation of DNA sequences, and high levels of this enzyme have been detected in cancer (36). In addition to full-length DNMT3B, more than 40 transcripts have been detected in common adult malignant tumors (18, 37). Many of the encoded proteins lack either the DNA binding or catalytic domains, and some have been shown to modify the pattern of DNA methylation and gene expression
In this study, we found DNMT3B7 transcripts in all of the ganglioneuromas tested and at very high levels in all of the ganglioneuroblastomas, leading us to the hypothesis that DNMT3B7 could induce differentiation and lead to a more benign phenotype, thereby modifying tumor phenotype in pediatric neuroblastoma.

To test this hypothesis, we forced DNMT3B7 expression in neuroblastoma cells using retroviral vectors to drive either constitutive or inducible expression and evaluated its effects on DNA methylation, tumor growth, and angiogenesis. We observed growth inhibition of aggressive neuroblastoma cell lines and xenograft tumors with slowed tumor growth and suppression of angiogenesis. Although aberrant hypermethylation of specific tumor suppressor genes and a CpG-methylator phenotype have been associated with clinically aggressive neuroblastomas (37), cancer cells are globally hypomethylated compared to normal cells. Thus, the increase in global DNA methylation observed in neuroblastoma cells with forced DNMT3B7 expression and decreased tumor growth are consistent with a non-malignant phenotype.

Previously, we have demonstrated altered DNA methylation and gene expression in 293 cells overexpressing DNMT3B7 (18). In addition, a re-distribution of DNA methylation was observed in DNMT3B7 transgenic mice (21). Forced expression of other DNMT3B isoforms containing the catalytic domain (DNMT3B1-4) have been shown to induce hypermethylation of repetitive elements (38). The truncated DNMT3B variants that lack DNA binding or catalytic domains are likely to have altered function compared with DNMT3B, and may actually stimulate the activity

![Figure 5. RNA-Seq validation and CpG island methylation. A, validation of overexpression of six genes in DNMT3B7-expressing cells by real-time RT-PCR. B, validation of underexpression of six genes in DNMT3B7-expressing cells by real-time RT-PCR. C, bisulfite sequencing of RXRB, a gene downregulated in DNMT3B7-expressing cells. D, bisulfite sequencing of EEA1, a gene upregulated in DNMT3B7-expressing cells.](image-url)
of other DNMTs with which it heterodimerizes (39), leading to hypermethylation.

GeneGo pathway analysis of the RNA-Seq data of the DNMT3B7-expressing LA1-55n cells identified several genes that may be important in neuroblastoma pathogenesis: KIF20B, ROCK1, ROCK2, APC and genes encoding members of the AP1 complex: FOSB, FOS, and JUNB. Increased expression of KIF1A has been found to correlate with high-risk neuroblastoma tumors (40). ROCK1 (six-fold) and ROCK2 (six-fold) were both underexpressed in DNMT3B7-expressing cells, and activated RhoA and its downstream effector ROCK have been shown to be negative regulators of growth cone motility (41).

Adenomatous polyposis coli (APC) is a tumor suppressor gene, highly expressed in the developing and adult nervous system, which is involved in neurite formation (42, 43), and apoptosis of neural crest cells (44).

Additionally, GeneGo pathway analysis of the RNA-Seq data revealed decreased expression of genes encoding the components of the AP1 complex, which can antagonize the activity of retinoic acid receptors through protein-protein interaction without requiring DNA binding (27). We therefore hypothesized that, with reduced levels of API, retinoic acid receptors would be more active in the DNMT3B7-expressing cells, allowing augmentation of differentiation by retinoid treatment. Indicative of increased differentiation, DNMT3B7-expressing LA1-55n cells following ATRA treatment (Fig. 6B). It has been shown previously that ATRA treatment of another MYCN amplified neuroblastoma cell line (SK-N-BE) leads to promoter hypomethylation (14), although we did not observe any significant global changes in DNA methylation after ATRA treatment (Supplementary Fig. S9). In the future, screening neuroblastoma tumors for DNMT3B7-expression may indicate particular patients for whom ATRA treatment might be particularly effective. DNMT3B exists in a protein complex with the mitotic chromatin condensation components, including HDAC1 and SIN3A (45). When unbound, retinoic acid receptors bind NCOR2, SIN3A, and HDACs, leading to target promoter repression, which can be disassociated upon ATRA binding (46, 47).

Altered histone modifications have also been found in neuroblastoma tumors and have correlated to tumor aggressiveness. Elevated levels of EZH2, the enzymatically active component of the Polycomb Repressor Complex 2 and H3K27me3 marks have been identified at the promoters of tumor suppressors CASZ1, RUNXI, NGFR (p75), and NTRK1 (TrkA) in undifferentiated, poor prognosis neuroblastoma (48). Additionally, following retinoic acid induced differentiation of neuroblastoma cells, EZH2 expression is decreased, consistent with decreased binding of EZH2 to retinoic acid inducible target genes (49). Retinoic acid treatment therefore, leads to a reduction in repressive histone marks in addition to the changes in DNA methylation described above. Therefore, future work may focus on testing whether histone modifications are altered in this system.
Our findings suggest that forced expression of DNMT3B7 in neuroblastoma cells is able to drive genome-wide DNA methylation. This is similar to the effects seen in lymphomas that arise in Eμ-Myc/DNMT3B7 transgenic mice (21). However, in neuroblastoma, the expression of DNMT3B7 is anti-tumorogenic (Fig. 3), whereas DNMT3B expression in Eμ-Myc lymphomas accelerates tumorigenesis. There are many possible explanations for this: The observations are made in different species and cell types. In addition, there are no other aberrant DNMT3B transcripts present in the DNMT3B7-Eμ-Myc lymphomas, whereas the human LA1-55n neuroblastoma cell expresses multiple DNMT3B transcripts. Truncated DNMT3B proteins may fine-tune the DNA methylation machinery within a cell dependent upon the other active DNMTs. These studies provide insight into understanding the molecular basis for the altered distribution of DNA methylation seen in virtually all human cancers. We hope that our discoveries will also provide a basis for novel diagnostic, prognostic, and therapeutic strategies that will be applicable to neuroblastoma and other types of tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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References

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