Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma

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Sotos syndrome is an autosomal dominant condition characterized by overgrowth resulting in tall stature and macrocephaly, together with an increased risk of tumorigenesis. The disease is caused by loss-of-function mutations and deletions of the nuclear receptor SET domain containing protein-1 (NSD1) gene, which encodes a histone methyltransferase involved in chromatin regulation. However, despite its causal role in Sotos syndrome and the typical accelerated growth of these patients, little is known about the putative contribution of NSD1 to human sporadic malignancies. Here, we report that NSD1 function is abrogated in human neuroblastoma and glioma cells by transcriptional silencing associated with CpG island-promoter hypermethylation. We also demonstrate that the epigenetic inactivation of NSD1 in transformed cells leads to the specifically diminished methylation of the histone lysine residues H4-K20 and H3-K36. The described phenotype is also observed in Sotos syndrome patients with NSD1 genetic disruption. Expression microarray data from NSD1-depleted cells, followed by ChIP analysis, revealed that the oncogene MEIS1 is one of the main NSD1 targets in neuroblastoma. Furthermore, we show that the restoration of NSD1 expression induces tumor suppressor-like features, such as reduced colony formation density and inhibition of cellular growth. Screening a large collection of different tumor types revealed that NSD1 CpG island hypermethylation was a common event in neuroblastomas and gliomas. Most importantly, NSD1 hypermethylation was a predictor of poor outcome in high-risk neuroblastoma. These findings highlight the importance of NSD1 epigenetic inactivation in neuroblastoma and glioma that leads to a disrupted histone methylation landscape and might have a translational value as a prognostic marker.

cancer | DNA methylation | epigenetics | histone | overgrowth

Sotos syndrome is an autosomal dominant condition characterized by physical overgrowth during the first years of life, a distinctive facial appearance, and learning disability (1, 2) with an increased incidence of malignant neoplasms (1–4). The distinctive head shape and size has led to Sotos syndrome sometimes being called cerebral gigantism (1, 2). Mutations in the nuclear receptor SET [su(var)3–9, enhancer-of-zeste, trithorax] domain containing protein-1 (NSD1) gene are found in patients exhibiting the clinical symptoms of Sotos syndrome (1, 2, 5). There are occasional individuals with NSD1 defects that overlap clinically with Sotos syndrome and other conditions such as Weaver syndrome (6, 7). The vast majority of NSD1 mutational mechanisms, including truncating, missense, and splice-site mutations and deletions, result in loss of function of the NSD1 protein (1, 2).

The NSD1 protein contains a SET domain and other functional domains, including plant homeodomain and proline-tryptophan-tryptophan-proline domains, both of which are involved in a protein–protein interaction (8). NSD1 has histone methyltransferase activity, demonstrated by the use of a recombinant protein-containing SET domain of NSD1 that has the ability to methylate the histone lysine residues H3-K36 and H4-K20 (9) and in the context of leukemia cells within the fusion protein NUP98-NSD1 to methylate H3-K36 in association with gene activation (10). There is growing evidence that deregulation of SET domain-containing proteins, such as NSD1, has an important role in cellular transformation (11), with the leukemia-translocated H3-K4 and H3-K36 histone methyltransferases hDOT1L and MLL, respectively, being further examples (12, 13). The case for NSD1 is even more interesting because its genetic disruption in Sotos syndrome is associated with an elevated risk of cancer (1–4), as occurs with other childhood overgrowth conditions such as Beckwith-Wiedemann (14). Because patients with NSD1 germ-line genetic disruption have an increased risk of developing malignancy before adulthood, including neuroblastoma, Wilms tumors, and hematological malignancies, a tumor-suppressor function for NSD1 might be proposed. This putative role is also supported by the presence of genomic rearrangements involving NSD1 in leukemias (10, 15) and breast cancer cells (16). However, somatic mutations of NSD1 have not been described in sporadic neoplasms. Transcriptional inactivation by cytosine/phosphate/guanine (CpG) island promoter hypermethylation is an alternative mechanism for the inactivation of tumor suppressor genes (17–19). Similar scenarios to that outlined for NSD1 have been described for familial tumor-suppressor genes, such as hMLH1 and BRCA1, which are very rarely mutated in sporadic tumors, but undergo epigenetic inactivation in noninherited neoplasms (17–19).

Here, we demonstrate that NSD1 undergoes CpG island promoter methylation-associated gene silencing in human neuroblastoma and glioma cells. NSD1 epigenetic inactivation is associated with global diminished levels of trimethylated histone lysine residues H4-K20 and H3-K36, and NSD1 protein absence in the 5′ end regulatory of its target genes, such as the oncogene MEIS1. Furthermore, the reintroduction of NSD1 reduces colony formation and cell growth, supporting a tumor-suppressor role. Most

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important, the presence of aberrant methylation at the NSD1 promoter predicts worse survival in high-risk neuroblastoma patients.

Results

NSD1 Promoter CpG Island Hypermethylation Leads to Gene Inactivation. NSD1 is a gene candidate for hypermethylation-associated inactivation in human cancer because a 5′-CpG island is located around the transcription start site (Fig. 1*A*). To analyze the DNA methylation status of the promoter-associated CpG island, we screened 72 human cancer cell lines from 12 different cell malignancy types (Table S1), using bisulfite genomic sequencing and methylation-specific PCR targeted to the area surrounding the transcription start site. NSD1 CpG island promoter hypermethylation was found in three cancer cell lines: LAI-5S (neuroblastoma), LAN-I (neuroblastoma), and U373-MG (glioblastoma) (Fig. 1 *A* and *B*). All normal tissues analyzed, including lymphocytes and brain, were completely unmethylated at the NSD1 promoter (Fig. 1*A* and *B*). Genomic sequencing of the NSD1 gene in unmethylated neuroblastomas and gliomas cell lines did not reveal any mutation (Fig. S1).

Having noted NSD1 promoter hypermethylation in cancer cell lines, we assessed the association between this epigenetic aberration and the putative transcriptional inactivation of the NSD1 gene at the RNA and protein levels. The cancer cell lines LAI-5S, LAN-I, and U373-MG, hypermethylated at the NSD1 CpG island, had minimal expression of the NSD1 RNA transcript, as determined by RT-PCR (Fig. 1*C*) and NSD1 protein, as determined by Western blot analysis (Fig. 1*D*) and immunofluorescence (Fig. 1*E*). In contrast, SK-N-JD and SK-N-BE(2)C, two neuroblastoma cells lines unmethylated at the NSD1 promoter, expressed NSD1 RNA and protein (Fig. 1 *C–E*). We established a further link between NSD1 CpG island hypermethylation and its gene silencing by treating the methylated cell lines with a DNA demethylating agent. The treatment of the LAI-5S, LAI-55N, LAN-I, and U373-MG cell lines with the demethylating drug 5-aza-2′-deoxycytidine restored the expression of NSD1 RNA transcript and protein (Fig. 1 *C* and *D*).

NSD1 Epigenetic Silencing in Cancer Cells Is Associated with Diminished Trimethylated-K20-H4 and Trimethylated-K36-H3. We next examined whether the loss of the NSD1 transcript by 5′-CpG island hypermethylation affected its histone residue marks, methylation of the histone lysine residues H3-K36 and H4-K20 (9, 10). Using Western blot analysis, immunofluorescence, and high-performance capillary electrophoresis, we observed reduced levels of trimethylated H3-K36 and H4-K20 in LAI-5S and LAN-1 cells (Fig. 2*A* and Fig. S1), all of them hypermethylated at the NSD1 promoter, compared with SK-N-BE(2)C and SK-N-JD cells (Fig. 2*A* and Fig. S1), the latter two having an unmethylated NSD1 promoter. Interestingly, the levels of dimethylated forms of H3-K36 and H4-K20 were similar in cancer cells with hypermethylated or unmethylated NSD1 CpG islands (Fig. S2), suggesting a more prominent role for NSD1 in establishing the trimethylated histone lysines residues. To reinforce the concept that NSD1 was the enzyme mainly responsible for the described histone trimethylated marks in our tumoral context, we analyzed the expression levels of other histone methyltransferases targeting these particular lysines, such as SET2 (HYPB/HIF-1, KMT3A) and SMYD2 (KMT3C), which specifically methylate H3-K36 (20, 21), and Pr-SET7/8 (KMT5A) and SUV4–20h1,h2 (KMT5B,C), which have been implicated in the monomethylation and trimethylation of H4-K20 (22, 23). We observed similar expression of these four enzymes in cancer cells with hypermethylated and unmethylated NSD1 (Fig. 2*B*), strengthening the evidence for the function of NSD1 in the described histone modifications. Furthermore, we extended the analyses of the trimethylated H3-K36 and H4-K20 forms to lymphoblastoid cell lines obtained from Sotos syndrome patients to determine whether they recapitulate the diminished levels of these marks observed in NSD1 epigenetically silenced cancer cells. The assessment of seven Sotos syndrome samples carrying different inactivating genetic disruptions of NSD1 showed that these patients indeed had significantly decreased trimethylated H3-K36 and H4-K20 (Fig. 2*C* and Fig. S1) compared with lymphoblastoid cell lines from healthy donors, again supporting a central role for NSD1 in the formation of the aforementioned trimethylated lysine residues.

Loss of NSD1 Recruitment to 5′-Regulatory Regions of Growth-Promoting Genes in Hypermethylated Cancer Cells: The Example of the MEIS1 Oncogene in Neuroblastoma. The divalent specificity to methylate H4-K20 and H3-K36 in vitro (9) is currently thought to be unique to NSD1, and our findings indicate that there is also double substrate specificity in vivo. H4-K20 methylation appears to be associated with transcriptional silencing (23, 24) and H3-K36 methylation has been found primarily in active genes throughout the gene body (25, 26), but it also might also be present in inactivated genes, in particular cellular contexts and genome loci (27). NSD1 is also implicated in transcriptional repression through its interaction with NIZP1, a zinc finger protein that interacts with the C5HCH domain of NSD1 and represses transcription in an NSD1-dependent fashion (28). Thus, it is possible that abrogation of NSD1-mediated repression of growth-promoting genes might contribute to human tumorigenesis.

To identify NSD1 target genes that might fit this candidate criterion, we used an RNA interference approach to deplete NSD1 expression in an unmethylated-expressing neuroblastoma cell line (SK-N-JD) (Fig. 3*A*) followed by expression microarray hybridization (GeneChip Human Genome U133 Plus 2.0 Array). The depletion of NSD1 expression was associated with lower cellular levels of the NSD1-related histone marks: trimethylated-K20-H4 and trimethylated-K36-H3 (Fig. 3*A*). The analysis of the expression microarray data from NSD1-depleted SK-N-JD cells demonstrated that 154 genes underwent a >2-fold change. Most of them [94 genes (61%)] underwent >2-fold up-regulation (Table S2) and 60 (39%) underwent >2-fold down-regulation (Table S2). Most importantly, among the up-regulated genes, one of the highest scorers with a fold-change value of 21.11 (Table S2) was the MEIS1 oncogene, a critical growth-promoting factor in neuroblastoma (29, 30).

Once we also confirmed the high up-regulation of the MEIS1 transcript upon NSD1 depletion by single quantitative RT-PCR (Fig. 3*B*), we performed conventional and quantitative ChIP for NSD1 occupancy at the 5′ regulatory region of the MEIS1 oncogene. We observed that the NSD1 protein was absent from the MEIS1 promoter region of NSD1 epigenetically silenced neuroblastoma cells, such as LAI-5S (Fig. 3*C*), but it did occupy the 5′ end regulatory region of MEIS1 in NSD1-expressing neuroblastoma cells, such as SK-N-JD (Fig. 3*C*). We also found a markedly reduced presence of the NSD1 protein in the MEIS1 promoter of Sotos syndrome lymphoblastoid cells with genetic disruption of the NSD1 gene (OGS55) (Fig. 3*C*), reinforcing the specificity of the experimental approach. Most importantly, we found that NSD1 recruitment to the MEIS promoter was associated with transcriptional repression of the MEIS1 gene (Fig. 3*D*), whereas neuroblastoma cells with NSD1 CpG island hypermethylation highly expressed the MEIS1 transcript (Fig. 3*D*) and protein (Fig. 3*D*) in association with the absence of NSD1 binding to the MEIS1 promoter. In a similar fashion, Sotos syndrome lymphoblastoid cells, which had an NSD1 genetic defect, expressed high levels of the MEIS1 transcript compared with healthy donors (Fig. 3*D*). Furthermore, NSD1 epigenetic silencing (LAI-5S) and genetic loss of NSD1 (OGS55) both are associated with a histone modification profile in the 5′ region of MEIS1 characterized by an enrichment in active marks (such as trimethyl-K4-H3) and a depletion in repressive marks (trimethyl-K20-H4, trimethyl-K9-H3, and trimethyl-K27-H3) (Fig. 3*E*). In contrast, unmethylated NSD1 neuroblastoma cells (SN-N-JD) show that NSD1 recruitment to the 5′ region of MEIS1 is

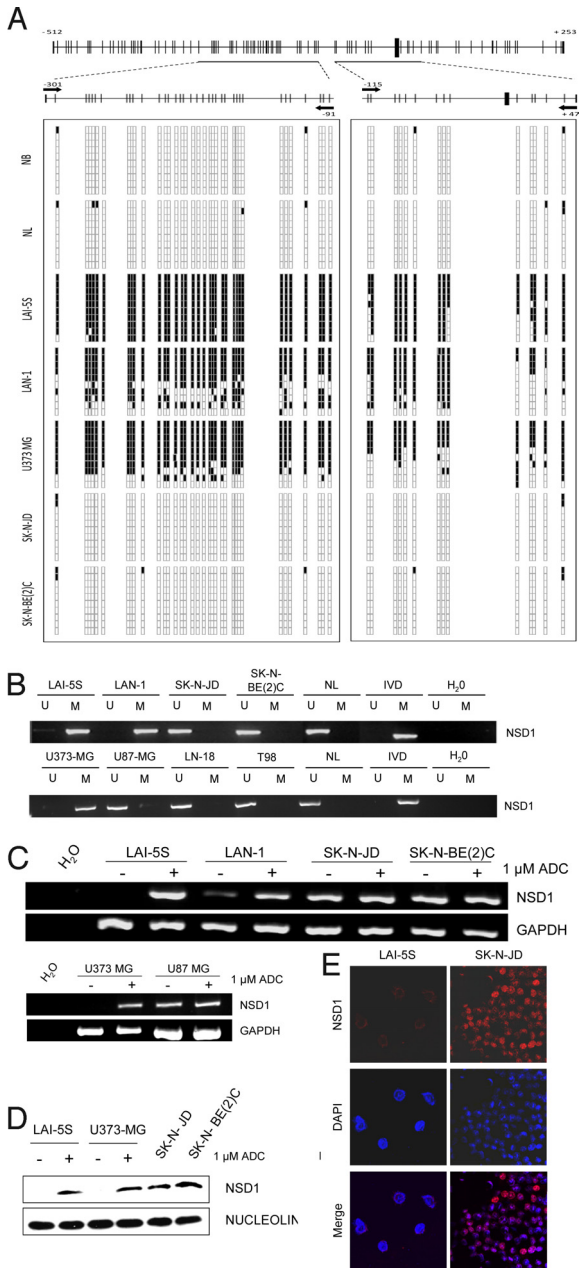

Fig. 1. Analysis of NSD1 CpG island promoter methylation status and gene function in human cancer cell lines. (*A*) Schematic depiction of the NSD1 CpG island around the transcription start site (long black arrow). CpG dinucleotides are represented as short vertical lines. Location of bisulfite genomic sequencing and methylation-specific PCR primers are indicated as black and white arrows, respectively. Results of bisulfite genomic sequencing of 10 individual clones in two regions of the NSD1 CpG island are shown. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. NL, normal lymphocytes; NB, normal brain. (*B*) Methylation-specific PCR for the NSD1 gene in cancer cell lines. The presence of a PCR band under lane M indicates methylated genes, while the presence under lane U indicates unmethylated genes. In vitro methylated DNA (IVD) is used as positive control. (*C*) RT-PCR analysis of NSD1 expression. Treatment with the demethylating agent (ADC+) reactivates NSD1 gene expression in the NSD1-hypermethylated cancer cell lines LAI-5S, LAN-1, and U373-MG. (*D*) Western blot analysis of NSD1 expression. The NSD1-hypermethylated cell lines LAI-5S and U373-MG do not express the NSD1 protein, in comparison with the unmethylated SK-N-JD and SK-N-BE(2)C cells. Treatment with the demethylating agent (ADC+) reactivates NSD1 gene expression. (*E*) Immunofluorescence analysis of NSD1 expression. The NSD1 CpG island methylated cell line LAI-5S does not stain for the NSD1 protein, in comparison with the unmethylated SK-N-JD cells. [Magnifications: 40× (LAI-5S) and 20× (SK-N-JD).]

associated with an enrichment in the transcriptional silencing histone marks (trimethyl-K20-H4, trimethyl-K9-H3, and trimethyl-K27-H3) and a depletion in the active marks (trimethyl-K4-H3) (Fig. 3*E*). Only one histone mark putatively associated with transcriptional activation is enriched when NSD1 is recruited to the 5′ region of MEIS1 (SN-N-JD), trimethyl-K36-H3 (also catalyzed by NSD1 as it occurs with its antagonistic silencing mark trimethyl-K20-H4) (Fig. S2), despite the minimal expression of the MEIS1 transcript (Fig. 3*D*) and protein (Fig. 3*D*). Thus, our results suggest that in neuroblastoma cells NSD1 promotes MEIS1 gene silencing by its trimethyl-K20-H4 repressor activity (9) so that it occurs in the presence of other histone marks of silencing (trimethyl-K9-H3 and trimethyl-K27-H3) and the loss of active marks (trimethyl-K4-H3). Together with the intriguing data derived from the trimethyl-K36-H3 ChIP assays, our findings confirm that NSD1 is a unique multitasked protein with a dual activity that can act as a repressor, as in our neuroblastoma model, or as a coactivator, as in the NUP98-NSD1 leukemia fusion protein (10), depending on the cellular context (31). Overall, our data link NSD1 protein promoter occupancy and the silencing of the MEIS1 oncogene and suggest that NSD1 epigenetic inactivation is an upstream mechanism that may well explain the observed overexpression of the MEIS1 oncogene in human neuroblastoma (30, 31).

NSD1 Has Tumor Suppressor-Like Properties in Cancer Cells. Related to the increased risk of malignancies in Sotos syndrome patients with NSD1 genetic disruptions (1–4) and our finding that NSD1 epigenetic silencing mediates the overexpression of the MEIS1 oncogene, we assayed the ability of NSD1 to function as a suppressor of tumor growth in our model, using the neuroblastoma cell line LAI-5S with NSD1 methylation-associated silencing. We first tested the inhibitory abilities of NSD1 in a colony-focus assay using G418 selection after transfection with the NSD1 gene (pEGFP-C1-NSD1) or the empty vector (pEGFP-C1). NSD1 expression was monitored by RT-PCR (Fig. 4*A*). NSD1 re-expression demonstrated tumor-suppressing activity with a reduction of 50% in colony-formation density with respect to the empty vector (Fig. 4*A*). We also measured the effect of transfection of NSD1 on the in vitro growth of LAI-5S cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay over time (Fig. 4*B*). Upon restoration of NSD1 expression in the LAI-5S hypermethylated neuroblastoma cell line, the cells proved less viable in the MTT assay (Fig. 4*B*). Because neuroblastoma is a tumor type where differentiation drugs, such as retinoic acid derivatives, have shown efficacy (32–34), we wondered whether one mechanism underlying growth suppression upon NSD1 transfection was the induction of differentiation. Neuroblastomas are composed of variable proportion of neuroblasts (neuronal lineage) and Schwannian-like cells (glial lineage) and neuroblastoma cell lines can be differentiated in these lineages (32, 33). We found that restoration of NSD1 expression in LAI-5S hypermethylated neuroblastoma cells induced glial differentiation assessed by the calcium binding protein S100B marker (Fig. S3), whereas neuronal differentiation determined by the neurofilaments markers NF68 and NF200 was not observed (Fig. S3). Finally, we also studied whether the knockdown of NSD1 expression by RNA interference in an expressing neuroblastoma cell line unmethylated at the NSD1 5′-CpG island, SK-N-JD, had growth stimulatory effects. We observed that the depletion of NSD1 expression in SK-N-JD cells, in which we also developed the expression microarray experiment described above, significantly increased cell growth (Fig. 4*C*). Overall, the data presented here suggest a tumor-suppressor role for NSD1.

NSD1 CpG Island Hypermethylation in Human Primary Malignancies: A Predictor of Poor Outcome in Neuroblastoma. After the demonstration of the epigenetic loss of function of NSD1 in cancer cell lines and its impact on the histone modification profile and tumor

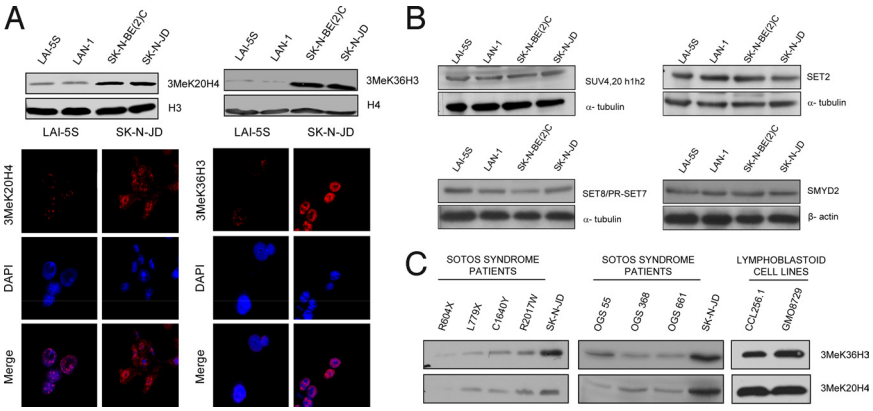

Fig. 2. Histone modifications and histone modifiers according to NSD1 status. (*A*) Western blot and immunofluorescence analysis of 3Me-K20-H4 and 3Me-K36-H3 in neuroblastoma cells. The NSD1-hypermethylated cell lines LAI-5S and LAN-1 show reduced levels of the 3Me-K20-H4 and 3Me-K36-H3 marks, in comparison with the unmethylated SK-N-BE(2)C and SK-N-JD cells. (Magnification: 40×.) (*B*) Western blot analysis of the histone modifiers SET2 and SMYD2 (methylation of H3-K36) and Pr-SET7/8 and SUV4–20h1,h2 (methylation of H4-K20) show similar expression with independence of the NSD1 epigenetic inactivation status. (*C*) Western blot analysis of 3Me-K20-H4 and 3Me-K36-H3 in Sotos syndrome samples. The lymphoblastoid cell lines from seven Sotos syndrome patients (R604X to OGS661) show reduced levels of the 3Me-K20-H4 and 3Me-K36-H3 marks, in comparison with unmethylated SK-N-JD neuroblastoma cells or lymphoblastoid cell lines from healthy donors (CCL256.1 and GMO8729).

growth, we assessed the prevalence of NSD1 CpG island promoter hypermethylation in cancer patients. We examined 377 human primary tumors corresponding to five different tissue types and observed an identical pattern with respect to the tumor type as that seen in the cancer cell lines. NSD1 CpG island hypermethylation was observed in primary neuroblastomas (37.9%, 69/182) and gliomas (11.1%, 8/72), but it was absent in other tumor types such as colon (0/49), breast (0/25), acute lymphocytic leukemia (0/34), and acute myelocytic leukemia (0/15) (Fig. 5*A*). NSD1 methylation status was confirmed by bisulfite genomic sequencing in 10 cases of primary neuroblastoma (five hypermethylated and five unmethylated) (Fig. S4). The presence of NSD1 CpG island hypermethylation in neuroblastomas was not associated with the methylation status of the described CpG island methylator phenotype (CIMP) targets in this tumor type (35–37), such as RASSF1A (Kendall's tau b test, $P = 0.948$), BLU ($P = 0.681$), PCDHG4C4 ($P = 0.292$), or CRYBA2 ($P = 0.519$) (Fig. S5). Most importantly, we observed that the presence of NSD1 hypermethylation in primary human neuroblastomas was associated with lower levels of the NSD1 transcript (Kendall's tau-b test, reverse correlation coefficient $r =$

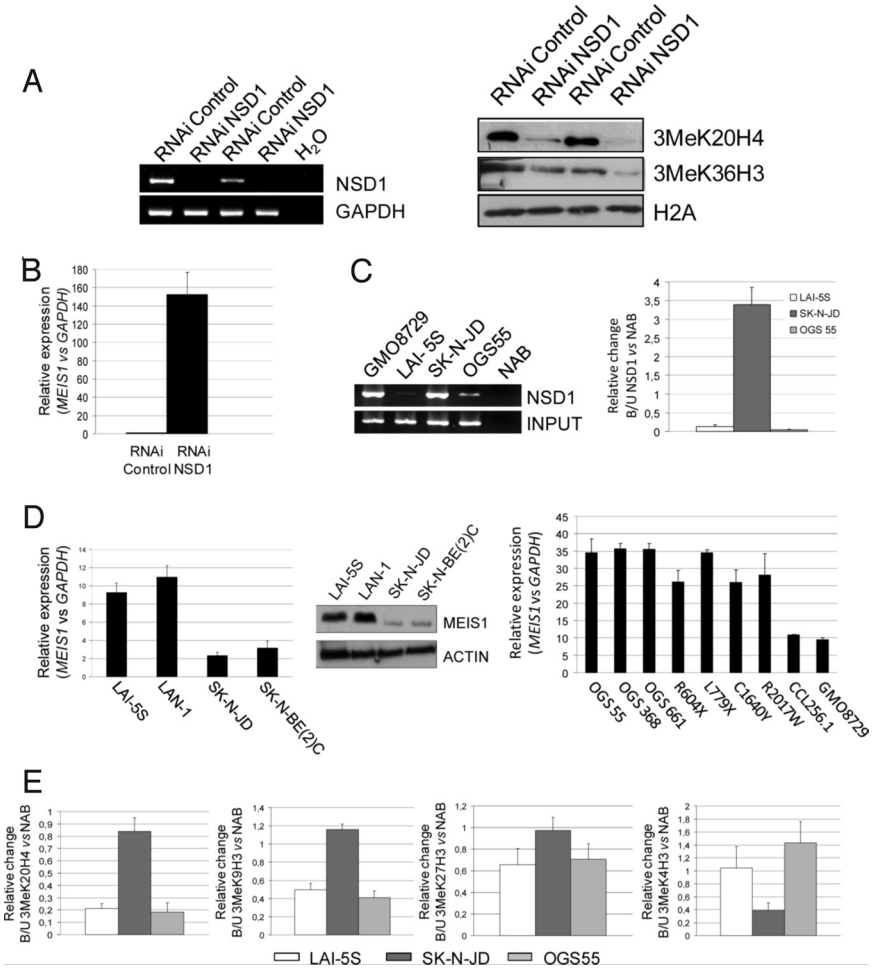

Fig. 3. Loss of NSD1 recruitment to the 5′ end of the MEIS1 oncogene in neuroblastoma. (*A*) (*Left*) NSD1 depletion by RNA interference in SK-N-JD cells is associated with reduced levels of 3Me-K20-H4 and 3Me-K36-H3. (*Right*) NSD1 expression upon RNA interference monitored by RT-PCR in CpG island unmethylated SK-N-JD cells. (*B*) Quantitative RT-PCR shows the high up-regulation of the MEIS1 transcript upon NSD1 depletion. (*C*) Determination by conventional and quantitative ChIP of NSD1 occupancy at the 5′ end promoter region of the MEIS1 gene. The presence of the NSD1 protein is evident in unmethylated neuroblastoma cells (SK-N-JD) and lymphoblastoid cells derived from healthy donors (GMO8729), whereas it is absent or extremely diminished in neuroblastoma cells with NSD1 epigenetic inactivation (LAI-5S) and lymphoblastoid cells from a Sotos syndrome patient (OGS55). (*D*) Quantitative RT-PCR and Western blot analysis shows the high up-regulation of the MEIS1 transcript in neuroblastoma cells with NSD1 CpG island hypermethylation (LAI-5S and LAN-1) compared with NSD1 unmethylated cells (SK-N-JD and SK-N-BE (2)C) (*Left*) and in Sotos syndrome lymphoblastoid cells (OGS55 to R2017W) compared with lymphoblastoid cells from healthy donors (CCL256.1 and GMO8729) (*Right*). (*E*) Quantitative ChIP for histone modification marks at the 5′ end of the MEIS1 gene. NSD1 epigenetic silencing (LAI-5S) and genetic loss (OGS55) both are associated with a depletion in repressive marks (3Me-K20-H4, 3Me-K9-H3 and 3Me-K27-H3) and an enrichment in active marks (3Me-K4-H3) compared with the NSD1 unmethylated SK-N-JD cells.

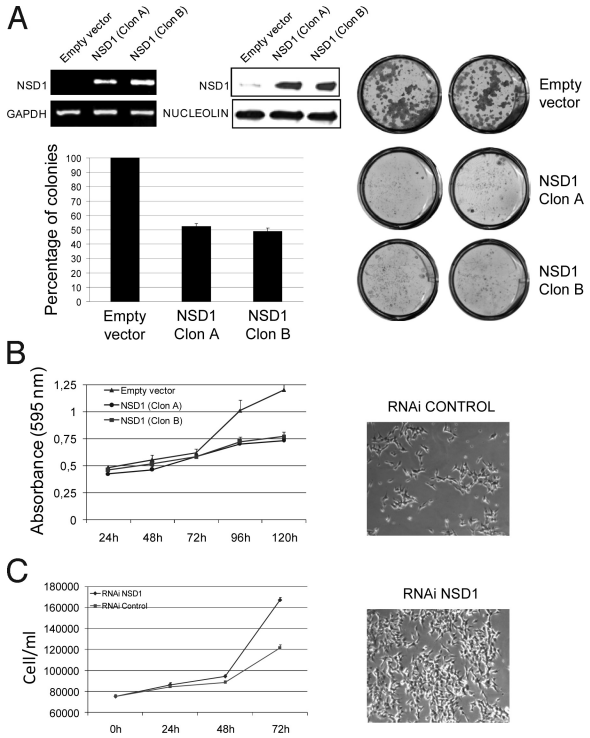

Fig. 4. Tumor suppressor-like properties of NSD1. (*A*) Colony formation assay. (*Left*) NSD1 expression monitored by RT-PCR and Western blot analysis in empty vector and NSD1-transfected LAI-5S cells and densitometric quantification of the colony formation density. (*Right*) Examples of the colony focus assay after 2-week selection with G418 and methylene blue staining. (*B*) A decrease of cell viability over time, determined by the MTT assay, upon NSD1 transfection is observed. (*C*) Effect of NSD1 depletion on cell growth in the NSD1 unmethylated SK-N-JD neuroblastoma cells. (*Left*) An increase of cell viability over time, determined by the MTT assay, upon NSD1 depletion is observed. (*Right*) Optical image of the effect of NSD1 reduction on the in vitro growth of SK-N-JD cells at 48 h. (Magnification: 10×.)

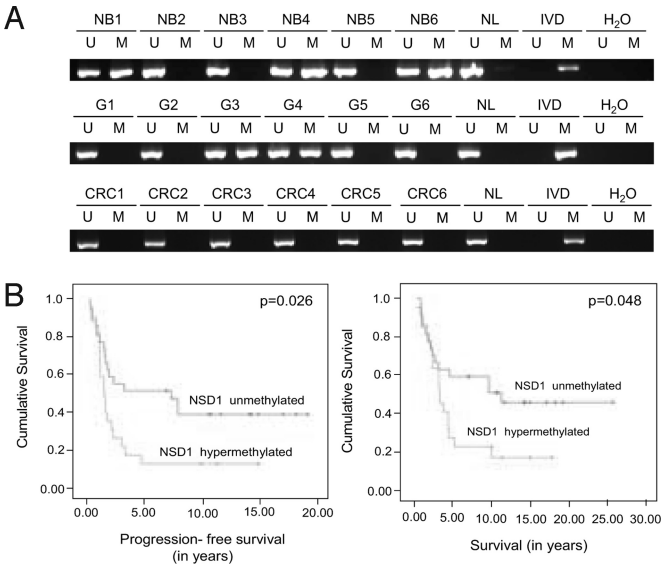

Fig. 5. NSD1 CpG island hypermethylation in primary human malignancies. (*A*) Analysis of NSD1 methylation by methylation-specific PCR. The presence of a PCR band under lane M indicates methylated genes, whereas the presence under lane U indicates unmethylated genes. Normal lymphocytes (NL) and in vitro methylated DNA (IVD) are used as negative and positive control for unmethylated and methylated genes, respectively. NSD1 hypermethylation is observed in primary neuroblastomas (N1-N6) and gliomas (G1-G6), but it was absent in other tumor types (example, colorectal cancer, CRC1-CRC6). (*B*) Kaplan–Meier analysis of NSD1 promoter hypermethylation in neuroblastoma patients. NSD1 promoter hypermethylation was significantly associated with lower overall survival ($P = 0.048$) and progression-free survival ($P = 0.026$).

-0.2368, $P = 0.0423$) (Fig. S5) using our generated mRNA expression microarray data (38).

Because of the substantial frequency of NSD1 hypermethylation observed in neuroblastomas, the most common extracranial solid cancer diagnosed during infancy and childhood (32) and with a mortality that approaches 30–60% overall (33), we wondered whether it had a correlation with clinicopathological and molecular features. We did not observe any association between NSD1 hypermethylation and age at diagnosis, clinical stage, age at death, presence of MYCN amplification, and loss of heterozygosity at the chromosomic regions 1p36, 1p22, 11q, 14q, 9p, 19q, and 17q G (Table S3). However, we found that the presence of hypermethylation of the NSD1 promoter influenced the survival of these patients. Of the 39 samples with gene hypermethylation, 17 (43.6%) were from surviving patients and 22 (56.4%) corresponded to deceased patients, whereas for the group of cases with the unmethylated gene, 41 (62.1%) cases survived and 25 (37.9%) died of the disease. The differences in these relative proportions were statistically significant (Fisher's exact test, $P = 0.015$), suggesting that the likelihood of death from disease was significantly higher in the group of tumors with NSD1 promoter hypermethylation. When all cases were considered, the Kaplan–Meier survival analysis carried out on hypermethylated and unmethylated tumor samples showed a nonsignificant trend for lower survival in the group of samples with a hypermethylated NSD1 gene ($P > 0.05$). However, the Kaplan–Meier survival analysis carried out on specific risk groups (Fig. 5*B*) demonstrated that NSD1 hypermethylation is a predictor of poor outcome in high-risk neuroblastoma, but not in low- or intermediate-risk neuroblastoma. In fact, the group of samples with NSD1 promoter hypermethylation showed significantly lower overall survival ($P = 0.048$) and progression-free survival ($P = 0.026$) than did the unmethylated samples. Thus, we can conclude that CpG island hypermethylation of the histone methyltransferase NSD1 is a significant predictor of poor outcome in high-risk neuroblastoma. This finding may be clinically relevant in the management of these patients.

Discussion

Overgrowth syndromes encompass a heterogeneous group of disorders that involve global, regional, or localized areas of excess growth relative to another part of the body or the age-related peer group. One striking feature of overgrowth syndromes is the risk of tumorigenesis (3, 4), but little is known about the potential contribution of the overgrowth-linked disease genes to sporadic human tumors. Herein, we show that epigenetic silencing of the NSD1 gene, responsible for the overgrowth Sotos syndrome, contributes to the development of nonhereditary neuroblastoma and glioma. These data match with the observation that in Sotos syndrome patients, who carry NSD1 genetic disruptions, there is a distinctive increased incidence of neural crest tumors, including neuroblastoma and glioma (3, 4).

The DNA methylation-associated loss of NSD1 in neuroblastoma and glioma provides double proof of the contribution of epigenetic alterations to human tumorigenesis, first by the lesion itself, the DNA hypermethylation event at the 5′ regulatory region of the gene, and second by the affected target gene, because NSD1 encodes a histone methyltransferase. Thus, NSD1 CpG island hypermethylation increases the growing body of data showing that the disruption of epigenetic genes is a common finding in human cancer. Illustrative examples are provided by the translocations of the histone methyltransferases MLL1 and hDOT1L (12, 13) or the mutations in the histone H3-K27 demethylase UTX (39) or the

microRNA processing machinery gene TARBP2 (40). In the case of NSD1, other SET2 family members such as NSD2 and NSD3 have also been reported to be genetically altered in cancer (11).

It is worth noting that before its identification as the gene for the Sotos syndrome NSD1 was already implicated in malignancy as a fusion partner for the NUP98 gene in childhood acute myeloid leukemia (AML) (10, 15). It is likely that the mechanism by which NSD1 contributes to AML differs from that controlling its tumorigenic role in Sotos syndrome patients and sporadic neuroblastomas and gliomas, because the NUP98-NSD1 fusion protein contains exons 6–23 that include almost all of the functional domains of NSD1 (41). AML does not occur more frequently in Sotos syndrome (3), and we have not found any leukemia with NSD1 CpG island hypermethylation. In addition, the NUP98-NSD1 fusion protein has been associated with gene activation (10), while we show at a candidate gene and global genomic level that NSD1 epigenetic silencing is linked to the activation of oncogenes, such as the paradigmatic case of MEIS1 in neuroblastoma. These data imply that NSD1 is a versatile protein that can act as a corepressor or coactivator, depending on the cellular context (31). Furthermore, it is worth noting that the loss of the trimethyl-K20-H4 enzymatic activity of NSD1 by its epigenetic silencing might explain, in the context of neuroblastoma and glioma, the commonly observed reduced levels of this histone mark in tumorigenesis (42).

Overall, our results demonstrate that epigenetic silencing of the histone methyltransferase NSD1 by promoter CpG island hypermethylation contributes to human cancer, provides a link between the loss of its histone methylation enzymatic activity and the activation of oncogenes, and supports the concept that the disruption of genes involved in overgrowth syndromes can also be relevant in the development of human sporadic tumors. Finally, these observations could have therapeutic implications because NSD1 DNA methylation-associated silencing is associated with poor outcome and there are now clinically approved DNA demethylating agents that may be able to restore NSD1 functionality with potential tumor growth inhibitory effects.

Materials and Methods

Full details are provided in *SI Text*. Briefly, DNA methylation status was established by PCR analysis of bisulfite-modified genomic DNA; NSD1 and MEIS1 expression was determined by semiquantitative RT-PCR, quantitative RT-PCR, and Western blot and immunolocalization analysis. NSD1 transfection was performed by electroporation and NSD1 knockdown by RNA interference. ChIP, colony formation, and cell viability assays were performed as described (40).

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