ZNF281 inhibits neuronal differentiation and is a prognostic marker for neuroblastoma

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Neuronal differentiation occurs through a complex array of epigenetic changes, posttranslational modifications, and the expression of specific genes. An example of the interplay between proteins and microRNAs is TAp73, a member of the p53 family (1, 2) that transcriptionally regulates the expression of miR34a in an axis necessary for the in vitro differentiation of cortical neurons and neuroblastoma (NB) cells (3, 4). Accord- ingly, the cortex and the hippocampus of TAp73 KO mice display developmental defects (3) and metabolic changes (5).

The zinc finger transcription factor Zfp281 is involved in the control of cell stemness by inhibiting Nanog expression in mice (6) through recruitment of the inhibitory complex NuRD on the Nanog promoter (7). Importantly, knock-out of Zfp281 in mice is embryonically lethal, suggesting a key role for this gene in development (6). However, this does not exclude other important functions performed by Zfp281 in adult cells. Indeed, ZNF281 (the human homolog of Zfp281) induces epithelial mesenchymal transition in colon cancer cells by controlling expression of SNAI1 and other key genes implicated in epithelial mesenchymal transition (8). In addition, ZNF281 knock-down promotes osteogenesis of multipotent stem cells (9). In line with this broader role of ZNF281 in somatic cells, we have recently demonstrated that ZNF281 is also involved in the regulation of the DNA damage response (10).

Here, using several experimental approaches, we show that ZNF281 is down-regulated during neuronal differentiation and negatively affects the differentiation process of murine cortical neurons and NB cells. High expression of ZNF281 defines a subset of patients with NB with poor clinical outcome. We also demonstrate that the expression of ZNF281 is induced by MYCN and inhibited by TAp73 acting through miR34a. In accordance with these data, we have identified neuronal differentiation-associated GDNF and NRP2 as two targets inhibited by ZNF281.

Results

ZNF281 Expression Is Down-Regulated During Neuronal Differentiation. Immature cortical neurons from embryos at day 17.5 post coitum (p.c.) were allowed to complete differentiation in vitro for 5 d (3, 4). Western blot analysis showed a sharp decrease in ZNF281 expression during the 5-d culture (Fig. L4). To further explore the behavior of ZNF281 during neural differentiation in man, we interrogated the R2 dataset that contains array-derived data on gene expression in human brains at different stages of embryonic and postnatal development. We demonstrated a significant decrease of ZNF281 expression comparing its expression levels before and after birth (Fig. 1B). To understand whether modulation of ZNF281 expression during the process of differentiation is also a common feature in transformed cells of neuro-ectodermal origin, we used NB cells that undergo neuronal differentiation on

Significance

High-risk neuroblastomas (NBs) show undifferentiated/poorly differentiated morphology as a distinctive feature. We have identified the transcription factor ZNF281 as a factor that can counteract the neuronal differentiation of primary neurons in culture and NB cells. The expression of ZNF281 is inhibited by TAp73 and promoted by MYCN. In turn, ZNF281 inhibits the expression of GDNF and NRP2, two proteins associated with neuronal differentiation. In patients with NB, the expression of ZNF281 is higher in high-risk patients and is associated with worse prognosis. Understanding the molecular mechanisms that regulate neuronal differentiation is relevant for the identification of defects in this process that underlie the development of tumors such as NB, in which an aberrant differentiation arrest has occurred.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE112029).

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treatment with retinoids and/or neurotrophins (11). Under basal conditions, ZNF281 was expressed at higher levels in the MYCN-amplified cell lines LAN-5, SK-N-BE (2), HTLA-230, and IMR32 compared with the MYCN nonamplified lines SK-N-SH, SIMA, and SH-SY5Y (12) (Fig. 1 C and D). When we induced neuronal differentiation in NB cell lines SH-SY5Y, LAN-5, SK-N-BE (2) by treatment with all-trans-retinoic acid (ATRA; 10 μM) for 8 days, we detected neurite outgrowth (SI Appendix, Fig. S1A4) and up-regulation of Neurofilament Medium Peptide (NEFM) (Fig. 1E) in ATRA-treated cells, as expected. Conversely, the expression of ZNF281 decreased throughout the differentiation process at both the protein (Fig. 1E) and transcript levels (SI Appendix, Fig. S1B). Decrease of ZNF281 expression during neuronal differentiation was also confirmed in other human NB cell lines (SK-N-AS, SK-N-SH, and IMR-32) (SI Appendix, Fig. S1C) and in the murine NB cell line NIE115 (SI Appendix, Fig. S1D).
**Appendix, Fig. S1 D and E).** Together, these results demonstrate a negative correlation between the expression of ZNF281 and the degree of neural differentiation in normal and transformed somatic cells.

**ZNF281 Affects Neuronal Differentiation of Murine Cortical Neurons and NB Cells.** To understand whether ZNF281 has a role in neuronal differentiation, we cultured murine cortical neurons at day 17.5 p.c. Cells were cotransfected with expression vectors encoding ZNF281 and a GFP-Spectrin fusion protein (pGFP-Spectrin) at a 15:1 ratio after 1 or 3 d in vitro (DIV1 and DIV3) to measure neurite length and number of dendrites. We detected a significant reduction in neurite length and branching in cells transfected at DIV1 with ZNF281/GFP-Spectrin compared with controls transfected with the empty vector/GFP-Spectrin after 48 h from transfection (Fig. 1F). Furthermore, cells transfected with ZNF281/GFP-Spectrin at DIV3 displayed reduction of neurite branching after 24 h from transfection (Fig. 1G).

To analyze whether the effects of ZNF281 on neuronal differentiation were also detectable in transformed cells, we transfected SH-SY5Y and LAN-5 NB cell lines with a pool of siRNAs that specifically knock-down the expression of ZNF281. We detected the onset of neuronal differentiation in cells transfected with siRNA against ZNF281 compared with control siRNA, as evaluated by neurite extension and increase of NEFM expression (Fig. 1H and I and SI Appendix, Fig. S2A). Furthermore, we labeled ZNF281-silenced SH-SY5Y cells with EdU to monitor their proliferation rate. FACS analysis of EdU-labeled cells did not detect a significant reduction in the percentage of cells in S phase between the ZNF281-silenced and the control cells, suggesting that the onset of differentiation driven by ZNF281 silencing is not a result of inhibition of proliferation (SI Appendix, Fig. S2 D and E). We confirmed the prodifferentiation effect of ZNF281 silencing by detecting an increase of NEFM expression and neurite outgrowth in the NB cell line LAN-5 infected with two lentiviral vectors encoding siRNAs directed against ZNF281 (Fig. 1J and K and SI Appendix, Fig. S2B). To further explore the role of ZNF281 in NB differentiation, we ectopically expressed ZNF281 in LAN-5 cells that were subsequently treated with ATRA (0.5 or 1 μM). At both concentrations, neurite extension and NEFM expression decreased in ZNF281 overexpressing ATRA-treated cells compared with control cells (Fig. 1L and M and SI Appendix, Fig. S2C), suggesting that ZNF281 expression hinders ATRA-induced neuronal differentiation. Altogether, these data imply a role of ZNF281 in maintaining an undifferentiated phenotype in murine cortical neurons and in NB cells.

**TAp73 Inhibits ZNF281 Expression Through miR34a.** Previous work has highlighted a role for TAp73 in inducing neuronal differentiation through the activation of miR34a (3, 4). Ectopic expression of TAp73 in the NB cell lines SH-SY5Y, LAN-5, and SK-N-BE (2) induced a decrease of ZNF281 (Fig. 2A and SI Appendix, Fig. S3A) and MYCN expression (Fig. 2A) and a parallel increase of miR34a expression (Fig. 2B). In colon cancer cells, miR34a posttranscriptionally inhibits the expression of ZNF281 (8). To test whether miR34a had a similar role in NB cells, we cotransfected the NB cell line BE2 (M17) with a luciferase reporter vector containing the 3′-untranslated region of the human ZNF281 gene and the pri-miR34a. Luciferase activity was significantly decreased in cells transfected with the premiR34a compared with those transfected with the control premiR (SI Appendix, Fig. S3B). Cotransfection of premiR34a and a reporter vector containing the 3′-untranslated region of ZNF281 in which the miR34a binding site was mutated did not cause any significant variation in luciferase activity compared with controls, demonstrating that miR34a posttranscriptional inhibition of ZNF281 in NB cells depends on the presence of a miR34a binding site in the 3′-untranslated region of ZNF281 (SI Appendix, Fig. S3B).

In keeping with these results, transfection of premiR34a in SH-SY5Y, LAN-5, and SK-N-BE (2) cells caused a decrease in ZNF281 expression, as opposed to an increase in NEFM (Fig. 2C), which further demonstrates the ability of miR34a to inhibit the expression of ZNF281 in NB cells. To evaluate whether there is a direct link among TAp73, miR34a, and ZNF281, we used the highly transfectable p53-null H1299 cell line (13) in which ZNF281 expression was reduced after transfection of TAp73β (SI Appendix, Fig. S3 C–E). We transfected H1299 cells with an anti-miR specific for miR34a (hereafter anti-miR34a) and, subsequently, with a plasmid encoding TAp73β. After 24 h, we detected a decrease of ZNF281 expression in cells transfected with TAp73β that was partially rescued by treatment with anti-miR34a (Fig. 2D). Of note, H1299 cells transfected with anti-miR34a (but not with TAp73β) showed an increase of ZNF281 expression as a result of the inhibition of the endogenous levels of miR34a (Fig. 2D). These data suggest a link among TAp73, miR34a, and ZNF281 that could be important in the control of neuronal differentiation.
Silencing of MYCN Inhibits ZNF281 Expression. To understand whether the oncogene MYCN could affect the expression of ZNF281 in NB cells, we transfected MYCN-amplified IMR32 and SK-N-VE (2) cells with siRNA against MYCN. The expression of ZNF281 was substantially inhibited in MYCN-silenced cells after 48 and 96 h (Fig. 2E) in parallel with an increase in miR34a expression (Fig. 2F). This result suggests that MYCN acts on ZNF281 through down-regulation of miR34a, similar to the action of c-Myc in colon carcinoma cells (8). In support of a role of c-Myc in controlling the expression of ZNF281 in NB cells, silencing of c-Myc in the MYCN nonamplified, c-Myc-expressing NB cell lines SH-SYSY, SK-N-SH caused a decrease of ZNF281 (SI Appendix, Fig. S3F). Conversely, ectopic expression of c-Myc or MYCN in SK-N-AS cells increased the expression of ZNF281 (SI Appendix, Fig. S3G).

GDNF and NRP2 Are Targets of ZNF281. To identify targets of ZNF281 associated with neuronal differentiation, we carried out a microarray analysis comparing gene expression of BE2 (M17) cells [a transfectable subclone derived from SK-N-VE (2) cells] (14) silenced with specific siRNA against ZNF281 with that of the same cells transfected with scrambled (scr) siRNA. Four biological replicates of each treatment were used for the microarray analysis. Technical details are given in Materials and Methods. We used a threshold of 1.2-fold up- or down-regulation to select RNAs differentially expressed in cells treated with siRNA against ZNF281 compared with siRNA scr controls. We detected 960 genes that are associated with several cellular processes (Fig. 3A). The efficacy of ZNF281 silencing was checked by Western blot analysis to detect the levels of ZNF281 protein in ZNF281-silenced cells and in scr controls (Fig. 3B). Among the modulated genes, 587 were up-regulated and 373 were down-regulated on ZNF281 silencing. Gene ontology classification highlighted 116 genes that were involved in nervous system development. We focused our attention on two of these genes for their direct involvement in neuronal differentiation: Glia-Derived Neurotrophic Factor (GDNF) and Neuropilin 2 (NRP2) play a role in inducing neuronal differentiation and in axon guidance, respectively (15, 16). We confirmed that GDNF and NRP2 expression are indeed up-regulated by ZNF281 silencing in independent experiments in BE2-M17 cells (Fig. 3C). Of interest, the expression of both genes is also up-regulated during ATRA-induced differentiation of BE2-M17 cells (Fig. 3D). Thus, ZNF281 is able to suppress the expression of genes that promote neuronal differentiation.

ZNF281 Binds to the Promoters of GDNF and NRP2. ZNF281 acts as a transcriptional repressor of Nanog by binding to its promoter (7). We hypothesized that ZNF281 could also bind to the promoters of GDNF and NRP2 to inhibit their expression. We analyzed the regulatory regions of both genes looking for GC-rich regions as potential binding sites for ZNF281 (8). We detected two CG-rich regions in the proximity of the transcription start site of GDNF and three near the transcription start site of NRP2 (Fig. 3E). We designed primers that included these regions to carry out a chromatin crosslinking immunoprecipitation (ChIP) analysis with a specific antibody for ZNF281. Results of these analyses clearly indicate binding of ZNF281 to the GDNF and NRP2 promoters (Fig. 3 F and G). We used Axin 2 as a positive control (10) and a region on chromosome 16 (16q22) as negative control (10) of ZNF281 binding (Fig. 3 F and G). These results suggest that ZNF281 inhibits the expression of GDNF and NRP2 through a direct mechanism.

Altogether our data indicate that ZNF281 in NB cells is positively controlled by MYCN and c-Myc, which in turn are repressed by TAp73, at least in part through miR34a; expression of ZNF281 inhibits NB differentiation by the negative regulation of differentiation-associated genes such as GDNF and NRP2 (Fig. 3H).

ZNF281 Expression Is a Prognostic Marker of NB. We used public datasets generated by microarray analyses of RNA from patients with NB to understand whether the expression of ZNF281 is compartmentalized in specific subsets of patients. We found that
the expression of ZNF281 was increased in stage 4 (metastatic, high-risk tumors) (17) compared with less aggressive stage 1 (Fig. 4A). ZNF281 expression is also elevated in high-risk (stage 4) compared with low-to-intermediate-risk patients (stages 1, 2, 3, and 4S; SI Appendix, Fig. S4A). Kaplan Meier analysis revealed that patients expressing high levels of ZNF281 have a significantly lower overall and event-free survival probability compared with low-expressing patients (Fig. 4B). Because the expression of ZNF281 is associated with MYCN amplification in NB cell lines (Fig. 1D) and MYCN amplification defines a subset of high-risk NBs, we sought to understand whether the expression of ZNF281 could have a prognostic value in subgroups of patients with or without MYCN amplification. Kaplan Meier analysis of patients without MYCN amplification indicated that high levels of ZNF281 expression predicts significantly lower overall (Fig. 4C) and event-free (SI Appendix, Fig. S4B) survival probability compared with low-expressors. Even in MYCN amplified patients, high expression of ZNF281 identified a subgroup of patients with worse overall and event-free survival probability (Fig. 4C and SI Appendix, Fig. S4B, respectively). In NB, clinical outcome is worse in patients >18 mo compared with those <18 mo of age (17). Kaplan Meier analyses in subgroups >18 mo and <18 mo of age highlighted a worse overall and event-free (SI Appendix, Fig. S4 C and D) survival probability for those patients expressing high levels of ZNF281 in both subgroups. We also evaluated whether the expression of ZNF281 could be prognostic in subgroups of NB patients >18 and <18 mo of age with or without MYCN amplification. Overall survival in all MYCN-amplified subgroups and event-free survival in MYCN-amplified >18 mo, in MYCN-amplified <18 mo, and in non-MYCN amplified >18 mo was worse in patients highly expressing ZNF281 (SI Appendix, Fig. S4 E and F).

Discussion

Here, we show that ZNF281 expression is associated with the undifferentiated state and decreases during terminal differentiation of murine cortical neurons and of NB cells induced by ATRA. The inverse association between ZNF281 expression and neural differentiation is further revealed by the decrease in ZNF281 levels that occurs in human brains from embryonic to adult life. In line with these observations, our data indicate that ZNF281 acts as a brake for the differentiation potential of murine cortical neurons and human NB cells, whereas its down-regulation triggers the onset of differentiation.

In an attempt to define mechanisms that govern ZNF281 expression in neural cells, we investigated the possible involvement of p73 (18, 19). In NB, where it is often expressed (3, 20), the TAp73 isoform plays a prodifferentiating role through its target miR34a (3). Our results indicate that TAp73 inhibits the expression of ZNF281, at least in part, through the posttranscriptional repression exerted by miR34a. Furthermore, we looked for activators of ZNF281 in NB cells. Here, MYCN plays a central role in controlling proliferation and differentiation of NB cells and tumors (21, 22). During NB differentiation, MYCN expression decreases and elevated MYCN levels are associated with aggressive and undifferentiated phenotype (23). MYCN expression is negatively regulated by TAp73 (24). We now show that inhibition of MYCN expression elicits a substantial reduction of ZNF281 expression. The latter observation suggests that MYCN promotes the expression of ZNF281 in NB cells. Thus, our data point to a control axis involving MYCN as a positive regulator of ZNF281 in contrast to TAp73, which negatively regulates its expression through its action on miR34a and through its inhibition of MYCN.

As a transcription factor, ZNF281 can affect the expression of several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibiting inflammation-associated genes through the recruitment of components of the NuRD complex on their regulatory regions (25). We envisaged that in neuroectodermally derived NB cells, ZNF281 could control several genes (6, 8) whose identity is most likely dependent on the cellular context. Indeed, recent data demonstrated that ZNF281 stimulates cardiac reprogramming by acting in association with GATA4 on cardiac enhancers and by inhibit.
GDNF is capable of inducing neuronal differentiation in many NB cell lines (15). NRP2, together with NRP1, plays an important role in gangliogenesis, axon guidance, and innervation of targets in the sympathetic nervous system (16). Functionally, NRP2 is a receptor for which SEMA3F is a ligand (16). Because NB cells derive from the neural crest as well as the sympathetic nervous system, it is conceivable that NRP2 plays a role in neurite outgrowth and, more generally, in neuronal differentiation in NB. GDNF and NRP2 together represent two examples of how ZNF281 can exert its inhibitory action of differentiation of NB cells by inhibiting the drivers of this process. The most common way through which ZNF281 inhibits its targets is through direct binding to their regulatory regions and the recruitment of inhibitory complexes of the NuRD type (7, 25). Our data demonstrate ZNF281 binding to GDNF and NRP2 promoters. In both cases, binding occurs in GC-rich regions very close to the transcription start site. These observations are consistent with a mechanism of transcriptional repression involving chromatin remodeling proteins.

NB derives from embryonic neuroectodermal cells that stop their differentiation process and remain in a proliferative phase. The causes of this differentiation block are not entirely understood, although the genomic amplification and the resulting overexpression of the MYCN oncogene play an important role (23, 27). More recently, the GPC2 gene, whose expression is promoted by MYCN or by the amplification of the GPC2 locus, was demonstrated to be essential for NB proliferation (28). Accordingly, GPC2 is mostly expressed in high-risk NB, but is generally absent in normal childhood tissues (28). Interrogation of two NB datasets indicates that the expression of ZNF281 is increased in patients with stage 4 NB whose tumors present less histologically differentiated features with respect to stage 1. Accordingly, patients with NB with high expression of ZNF281 have a poor clinical outcome compared with low-expressors. These observations on patient-derived samples are in agreement with a primary role of ZNF281 on the differentiation control of NB that we demonstrated with our experimental results.

In brief, our data suggest that ZNF281 acts in maintaining the undifferentiated state of normal and transformed neural cells. In NB cells, the expression of ZNF281 is controlled through an axis involving TAp73, miR34a, and MYCN. The inhibitory activity of ZNF281 on NB differentiation is exerted by the negative control of the expression of differentiation-associated genes such as GDNF and NRP2. Finally, the enhanced expression of ZNF281 in advanced stages of NB should be further evaluated as a prognostic factor of this disease.

Materials and Methods
Primary cortical neurons cultures were prepared from embryonic day 17.5 murine embryos, as previously described (4). Cortical neurons were cotransfected at the indicated day of culture, with an expression vector containing the coding region of ZNF281 (pcDNA3-ZNF281HA) and a vector expressing a GFP-Spectrin fusion protein (GFP-Spectrin) at a 15:1 ratio, using Lipofectamine 2000 (Invitrogen), as previously described (4). We analyzed the total dendritic length and branch number of each individual GFP-positive neuron, as previously described (4). Images were collected using a Leica DMI6000B digital inverted microscope (LEICA microsystem) and analyzed with LAS AF software, and immunofluorescence analysis is provided in SI Appendix, SI Materials and Methods. Uncropped images from WB and ChIP analyses are shown in SI Appendix, Figs. S5 and S6.