DNA methyltransferase 3B regulates duration of neural crest production via repression of *Sox10*

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Edited by Joseph R. Ecker, Howard Hughes Medical Institute and The Salk Institute for Biological Studies, La Jolla, CA, and approved November 5, 2014 (received for review September 30, 2013)

Neural crest stem cells arise within the central nervous system but then undergo an epithelial-to-mesenchymal transition to migrate away and contribute to the peripheral nervous system and craniofacial skeleton. Here we show that DNA methyltransferase 3B (DNMT3B) is responsible for the loss of competence of dorsal neural tube cells to generate emigrating neural crest cells. DNMT3B knockdown results in up-regulation of neural crest markers, prolonged neural crest emigration, and subsequent precocious neuronal differentiation of the trigeminal ganglion. We find that DNMT3B binds to the promoter of *Sox10*, known to be important for neural crest emigration and lineage acquisition. Bisulfite sequencing further reveals methylation of the *Sox10* promoter region upon cessation of emigration in normal embryos, whereas this mark is reduced after DNMT3B loss. Taken together, these results reveal the importance of DNA methylation in regulating the ability of neural tube cells to produce neural crest cells and the timing of peripheral neuron differentiation.

neural crest | epigenetic | DNMT3B | Sox10 | DNA methylation

The neural crest is a multipotent stem cell population, unique to vertebrates, that contributes to a wide variety of derivatives, including sensory and autonomic ganglia of the peripheral nervous system (PNS), cartilage and bone of the face, and pigmentation of the skin. Neural crest progenitors arise at the neural plate border, and after neurulation reside within the dorsal aspect of the central nervous system (CNS). They then undergo an epithelial-to-mesenchymal transition (EMT) and delaminate from the neural tube as migratory mesenchymal cells that navigate to diverse and sometimes distant locations. The timing of onset and cessation of neural crest emigration from the CNS is stereotypic. In birds, neural crest cells initiate EMT shortly after neural tube closure and cease emigration 1 d later. Control of proper neural crest production and migration is critical for normal development, with dysregulation of these processes leading to birth defects and peripheral neuropathies.

Initiation of neural crest emigration has been well studied. At trunk levels, it depends on appropriate levels of BMP signaling to regulate the G1/S transition of emigrating neural crest cells in a *Wnt*-dependent manner (1). At the level of transcription, *Snail2*, *Sox10*, and *Sip1* play important roles in EMT in both neural crest and cancer cells (2–4); for example, overexpression of Sox10 causes ventral neural tube cells to aberrantly undergo EMT and become migratory (5). In contrast, the mechanisms that restrict neural crest production over time are poorly understood. Single cell lineage analysis has shown that individual precursor cells can give rise to both neural crest and neural tube derivatives (6, 7). This raises the intriguing possibility that unknown factors, such as epigenetic modifiers, may progressively limit the competence of presumptive CNS cells to produce neural crest cells, thereby influencing the balance of neural tube vs. neural crest cell fate.

Consistent with this possibility, we previously showed that a DNA methyltransferase (DNMT3A) functions early to repress neural genes *Sox2* and *Sox3* in the presumptive neural crest, as a prerequisite for neural crest specification (8). DNA methyltransferases DNMT3A and 3B are essential for de novo methylation by catalyzing the transfer of a methyl group to cytosine residues on DNA (9). Both play important roles in development and disease (10, 11). DNMT3B null embryos exhibit rostral neural tube defects and growth impairment (12). Moreover, mutations in human DNMT3B are found in immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome, comprising facial abnormalities, neurologic dysfunction, and other defects (13) suggestive of abnormal neural crest development. Similarly, DNMT3 knockdown in zebrafish causes defects in craniofacial structures and improper neurogenesis (14). DNMT3B loss in human ES cells accelerates neural and neural crest differentiation and increases the expression of neural crest genes (*Pax3/7*, *FoxD3*, *Sox10*, and *Snail2*) (15). Taken together, these data are consistent with an important role for DNMT3B in neural crest development.

Here we tested the developmental function of DNMT3B in avian embryos, owing to their accessibility for transient knockdown and similarity to humans at early stages of development. Our results indicate that loss of DNMT3B prolongs neural crest production by the neural tubes and up-regulates the expression of key neural crest transcription factors such as *Sox10*. We show that DNMT3B directly binds to and methylates the *Sox10* promoter in neural tube progenitors, resulting in cessation of neural crest EMT. Thus, DNMT3B is involved in regulating the duration of neural tube cell competence to produce migratory neural crest cells.

Significance

Mutations of DNA methyltransferase 3B (DNMT3B) result in facial abnormalities and neurologic defects in humans related to abnormal neural crest development. To address the underlying mechanism, we tested the role of DNMT3B using loss-of-function and molecular analyses in chick embryos. Neural crest cells initially form within the central nervous system (CNS), then migrate away to form elements of the facial skeleton and peripheral nervous system (PNS). Our results show that DNMT3B acts by directly methylating the promoter region of the neural crest gene *Sox10* in neural tube progenitors, thereby inhibiting its transcription. This in turn influences the duration of neural crest production by the CNS, such that DNMT3B loss causes overproduction of neural crest cells and premature neuronal differentiation in the PNS.

Author contributions: N.H., P.H.S.-M., M.S.-C., and M.E.B. designed research; N.H., P.H.S.-M., M.S.-C., and E.S.-V. performed research; N.H., P.H.S.-M., M.S.-C., E.S.-V., and M.E.B. analyzed data; and N.H., P.H.S.-M., and M.E.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1318408111/-/DCSupplemental.

Results

DNMT3B Expression During Neural Crest Induction and Migration. We identified several epigenetic factors, including DNMT3B, in screens for neural crest genes (16, 17). To assess its functional significance, we first examined DNMT3B transcript expression from gastrulation to the formation of neural crest derivatives, focusing on the cranial neural crest region in chicken embryos. The results show that *Dnmt3b* is expressed broadly at gastrula stages throughout the neural plate and its border where neural crest cells are induced (Fig. 1). In addition, *Dnmt3b* is subsequently expressed in the dorsal neural tube and migratory neural crest. Initially expressed throughout the neural tube at stage 8, it becomes restricted to the dorsal portion by stage 10. In migrating neural crest cells, *Dnmt3b* colocalizes with HNK-1 immunostaining.

Loss of DNMT3B Causes Prolonged Neural Crest Production by Neural Tubes Without Affecting Specification. As its paralog DNMT3A (8) affects neural crest specification, we first examined whether DNMT3B acted similarly. Using two different fluorescein-tagged antisense morpholino oligomers (3B MO and 3B MO2) to block translation of DNMT3B protein, we examined the effects of its loss of function on early neural crest gene expression. Morpholinos were electroporated onto one side of stage 4 embryos, with the contralateral side serving as an internal control. No changes were noted in expression of neural crest marker *Sox10* shortly after neural tube closure, suggesting that loss of DNMT3B had no effect on initial neural crest specification or early migration (Fig. S1*A*).

To test whether DNMT3B might play a later role, we performed a similar experiment but at later stages. Morpholinos were introduced into one side of the embryo in ovo at stage 8, as the neural folds were elevating, and the effects on neural crest markers *Sox10* and *Snail2* during migratory stages were evaluated. As before, no alterations were observed at the onset of neural crest migration (stages 9–10; Fig. S1*A*); however, toward the end of migration (late stages 11–12), loss of DNMT3B appeared to cause excess and/or prolonged emigration of neural crest cells from the neural tube. Under normal conditions, *Sox10* and *Snail2* come on shortly before the initiation of neural crest migration and are maintained on migrating neural crest cells, and are then turned off in the dorsal neural tube after stage 11, concomitant with cessation of emigration. After DNMT3B knockdown, however, *Sox10*- or *Snail2*-positive neural crest cells appeared to continue emerging from the morpholino-treated side of the embryo but not from the control side, where they were observed only some distance away from the neural tube (Fig. 2 *A*, *D*, and *F*). In contrast, similar injections of control morpholino (Con MO) resulted in equivalent migratory patterns on both sides of the embryo, with cessation of emigration by stage 12 (Fig. 2 *A*, *C*, and *E*). *Sox2* and *Sox3* apparently were unaffected by DNMT3B knockdown (Fig. S1*B*).

To demonstrate specificity, we performed rescue experiments in which DNMT3B morpholino together with an expression vector containing a DNMT3B coding sequence were introduced at stage 8 (Fig. 2 *B* and *D*). Using 1.0 μg/μL of DNMT3B expression construct coelectroporated with 3B MO significantly ($P < 0.05$) rescued the phenotype compared with 3B MO plus empty vector. Using a lower concentration (0.5 μg/μL) of DNMT3B construct plus 3B MO led to a slight, but not significantly different ($P = 0.38$), improvement in severity (Fig. 2 *B* and *D*).

To definitively test whether neural crest emigration was prolonged, we labeled neural tubes with the lipophilic dye DiI after normal termination of cranial neural crest cell emigration (stage 12). DiI labeling of control MO-treated neural tubes at this stage yielded DiI label confined to the neural tube (Fig. 2*F*) and not present in migrating neural crest cells at midbrain/rostral hindbrain levels. In contrast, in 3B MO-treated embryos, DiI-labeled migrating neural crest cells continued to emigrate from the neural tube at these axial levels on the MO-injected side (Fig. 2*F*). A few DiI-labeled cells were seen on the contralateral side as well, likely representing cells generated from the MO-treated side that crossed the midline, given that ∼20% of neural crest cells "cross" to the opposite side (6). These results demonstrate that DNMT3B loss leads to extended production of cranial neural crest cells beyond the time when emigration normally would cease in control embryos.

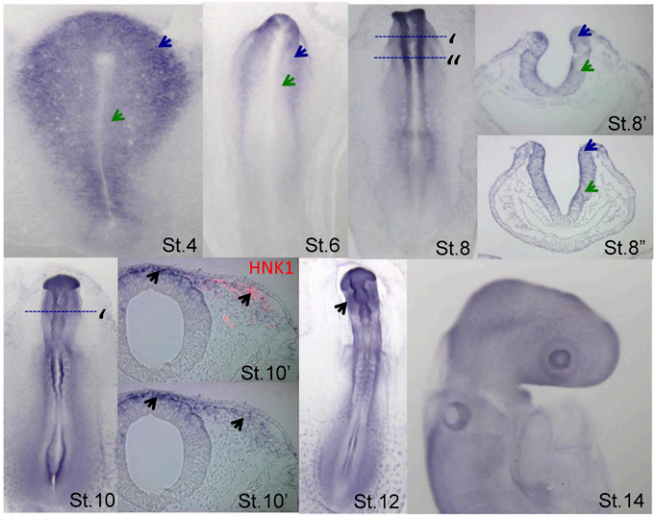

Fig. 1. DNMT3B is expressed in the neural crest territory. Shown is the expression pattern of DNMT3B in stage 4–14 chicken embryos by in situ hybridization. During stages 4–8, DNMT3B is expressed throughout the neural plate (green arrowhead) and neural plate border (blue arrowhead). During migratory stages (stages 10–11), expression of DNMT3B is restricted to the dorsal neural tube and migratory neural crest cells (black arrowhead). Using HNK-1 staining (red) as a marker for migrating neural crest cells, DNMT3B colocalizes with HNK-1–positive migrating neural crest cells as well as in neural crest derivatives. DNMT3B is no longer expressed at stage 14. The dashed line indicates the level of cranial region cross-sectioned.

Multiplex Analysis Shows that Loss of DNMT3B Up-Regulates Neural Crest Specifier Genes, While Down-Regulating Dorsal Neural Tube Genes. To consider global changes in gene expression caused by DNMT3B knockdown, we performed multiplex NanoString analysis to simultaneously monitor and quantify 70 transcripts involved in neural crest and tube development, as well as cell proliferation and cell death. NanoString is exquisitely quantitative because it does not require RNA extraction or enzymatic reaction, thus allowing accurate quantitation of transcript levels.

Individual half dorsal neural tubes from DNMT3B knockdown embryos were analyzed at stage 12 by comparing the morpholino-electroporated and uninjected sides of the same embryo (Fig. 3*A*). Given that there are no significant differences between the two sides of Ctrl-MO–treated embryos (4, 18) with a <20% variation, we defined 30% variation as our cutoff for statistical significance (Fig. 3*A*, red and green dotted lines). DNMTs are thought to inhibit transcription by methylating the promoter region of target genes; thus, genes up-regulated as a consequence of DNMT3B loss represent potential direct targets.

NanoString data analysis averaged over six embryos showed that neural crest specifier genes *TfAp2A*, *Sox9*, *Sox10*, *Snail2*, and *FoxD3* were all significantly up-regulated as a result of DNMT3B knockdown (Fig. 3*B*). In contrast, *Ncad* was down-regulated (Fig. 3*B*), and expression of cell proliferation (*Pcna*), cell death (*Perp* and *Bcl2*), and housekeeping (*Eef1A1*, *Gapdh*) genes was unchanged between the injected and uninjected sides.

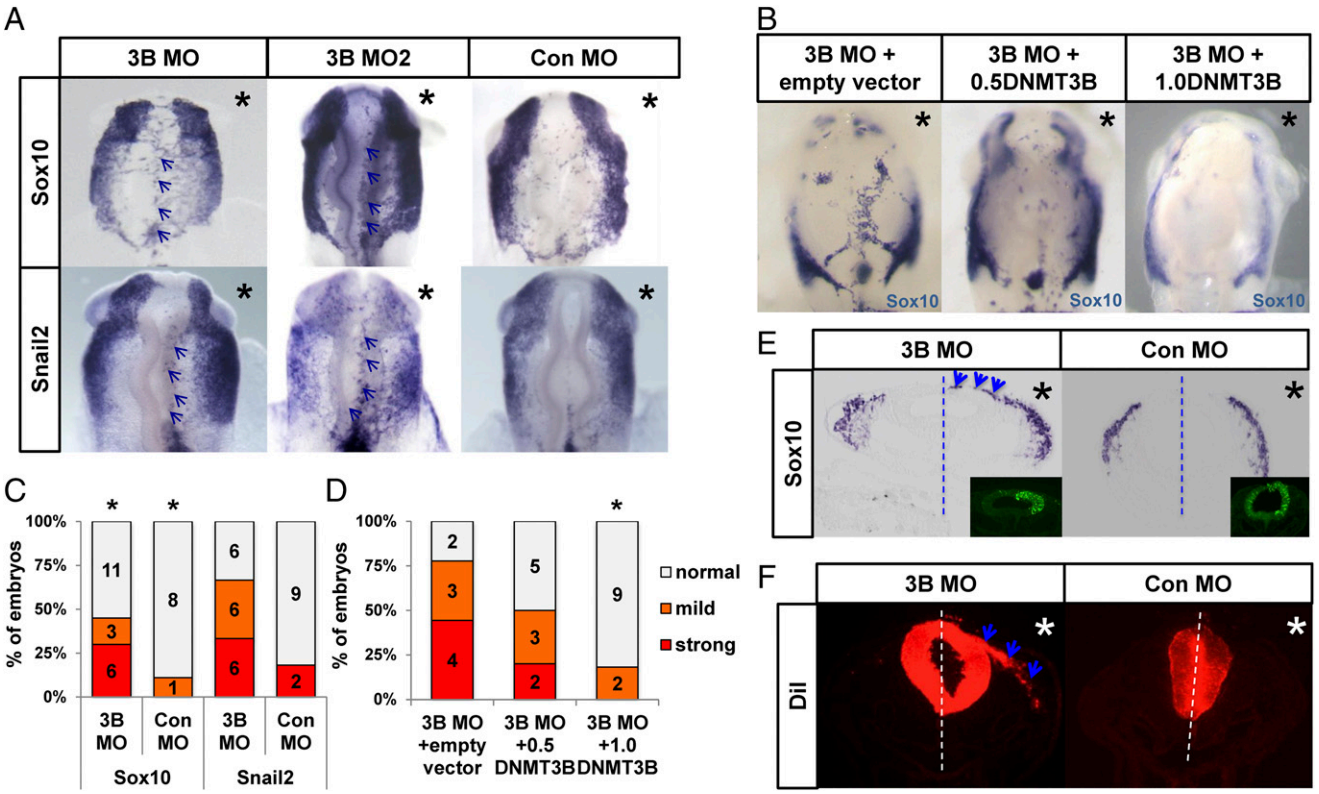

Fig. 2. Loss of function of DNMT3B causes prolonged migration of neural crest cells using *Sox10*, *Snail2*, and DiI as markers. (*A*) Electroporation of morpholinos against DNMT3B (3B MO and 3B MO2) caused prolonged emigration of neural crest cells compared with control morpholino (Con MO). Cranial neural crest cells marked by *Sox10* or *Snail2* appear to keep emigrating from the neural tube for longer periods (arrow) on the side electroporated with 3B MO compared with the internal control side. Embryos electroporated with Con MO show similar expression on both sides. (*B*) Rescue experiments were performed by coelectroporating 3B MO with two different concentrations of the overexpressing vector containing the DNMT3B coding sequence (0.5DNMT3B and 1.0DNMT3B). Effects were analyzed by in situ hybridization for detection of *Sox10* expression. An asterisk indicates the side of injection. (*C*) Quantitation of percentage of 3B MO- or Con MO-treated embryos with either a strong or a mild prolonged emigration phenotype using *Sox10* or *Snail2* as markers. (*D*) Quantitation of the phenotype of rescue experiments of 3B MO plus empty vector, 0.5 μg/μL DNMT3B (0.5DNMT3B) or 1.0 μg/μL DNMT3B vector (1.0DNMT3B). Treated embryos were characterized as having a strong or a mild prolonged emigration phenotype using the *Sox10* marker. $*P < 0.05$, χ^2 contingency test. Numbers in the graphs represent the numbers of analyzed embryos. (*E*) Transverse sections of embryos treated with 3B MO and those treated with Con MO. *Sox10*-positive neural crest cells continue to emigrate out of the neural tube (arrows) on the 3B MO-treated side compared with the internal control side. In Con MO-treated embryos, emigration has ceased on both sides. (*Inset*) FITC with morpholino incorporation. An asterisk indicates the side of MO injection. (*F*) Embryos were electroporated with MO at stage 8 into the right side of the neural tube (asterisk), and DiI was used to label the entire neural tube during early stage 12, by which time emigration ceased in normal embryos. Embryos were collected and sectioned at stage 13. Prolonged emigration was observed (arrows) on the side treated with 3B MO, whereas no emigration was observed on the uninjected side or the Con MO-treated side. Four of the nine embryos treated with 3B MO and none of the seven embryos treated with Con MO had a prolonged emigration phenotype.

Loss of DNMT3B Results in Excess Migrating Neural Crest and Premature Differentiation of Trigeminal Ganglia. To examine later effects caused by loss of DNMT3B, we collected embryos at stages of neural crest migration and examined expression of the migratory neural crest marker HNK-1. Compared with the control side of the same embryo, the DNMT3B knockdown side had many more neural crest cells, as observed in both whole-mount and transverse sections (Fig. 4*A*). Quantification of the increase in migratory neural crest cells using two concentrations of 3B MO showed significant differences compared with control MO treated embryos (Fig. 4*B*). Similarly, excess *Sox10*-expressing cells were detected by in situ hybridization on the DNMT3B knockdown side at stage 13 (Fig. 4*C*).

DNMT3A and 3B paralog expression patterns have some overlap, raising the possibility that they may act in a functionally redundant fashion. However, knockdown of DNMT3A alone had little effect on neural crest emigration when introduced at stage 8 (Fig. 4*D*). In contrast, dual knockdown of DNMT3A and 3B had profound effects on neural crest production (Fig. 4*D*), comparable to or even more penetrant than those observed with 3B MO alone. This suggests that the two paralogs are likely to play temporally distinct roles, with DNMT3A primarily acting early (8) and DNTM3B acting toward the end of neural crest emigration.

To examine effects on neural crest differentiation, we stained embryos with TUJ1 as a marker of neuronal differentiation. Surprisingly, we observed early condensation of the trigeminal ganglion and premature neuronal differentiation on the 3B MO-treated side of the embryo (Fig. 5). Whereas neuronal differentiation was just beginning on the uninjected side or in control morpholino at late stage 13, well-differentiated and condensed trigeminal ganglia with obvious ophthalmic and maxillo-mandibular lobes were present on the 3B MO-treated sides.

DNMT3B Binds to and Methylates the *Sox10* Promoter. Our NanoString and in situ data raise the intriguing possibility that DNMT3B could regulate de novo methylation and subsequent repression of genes involved in the maintenance of neural crest progenitors and EMT, thus preventing continuous neural crest formation. CpG island promoters were recently shown to be largely hypomethylated, even when silenced on different tissues and developmental stages in many vertebrate models (19); thus, we examined the presence of CpG islands in the promoter

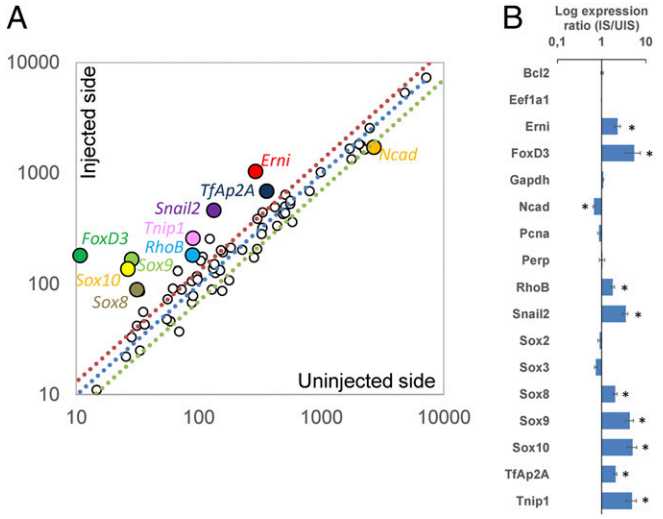

Fig. 3. Multiplex NanoString analysis shows that the neural crest specifier genes *Snail2*, *Sox9/10*, and *FoxD3* are up-regulated on loss of DNMT3B, whereas Ncad is down-regulated. (*A*) NanoString analysis of each half dorsal neural tube from a representative embryo revealing genes up-regulated and down-regulated by DNMT3B knockdown. The injected side of 3B MO-treated embryos shows >25% up-regulation (above the red dotted diagonal line) of neural crest specifier genes *Sox10*, *Sox9*, *Snail2*, *TfAp2A*, and *FoxD3*, and >25% down-regulation (below the green dotted diagonal line) of type I cadherin *Ncad*. (*B*) Bar graph showing the mean ± SD log expression ratio (between injected and uninjected sides) of six representative 3B MO-treated embryos. Asterisks indicate significant differences ($P < 0.05$) on the injected side compared with the uninjected side, analyzed using the Student *t* test.

regions of neural crest specifier genes up-regulated after DNMT3B loss. Interestingly, only *Sox10* lacks CpG islands in its promoter region, whereas these islands are present in other neural crest specifier genes, including *Snail2* and *FoxD3* (Fig. S2). Thus, *Sox10* is a likely candidate for promoter methylation by DNMT3B.

To test this idea in vivo, we used microChIP (18), followed by quantitative PCR (qPCR), to assess whether *Sox10* is a direct target of DNMT3B. To this end, embryos were electroporated with a construct encoding full-length DNMT3B tagged with FLAG at the N terminus. After development to stage 12, dorsal neural folds were dissected and processed for microChIP. The data show that DNMT3B associates with the promoter region of *Sox10*, compared with gene desert regions of chromosomes 1 and 2 serving as control regions (Fig. 6*A*). In contrast, the *Snail2* promoter exhibited only a low level of occupation with DNMT3B compared with *Sox10*. Similarly, DNMT3B failed to bind to the regulatory region of *FoxD3* (Fig. S3), suggesting that *Snail2* and *FoxD3* are indirect targets. These findings definitively show that DNMT3B directly binds to and represses the promoter region of *Sox10*, thereby shutting down neural crest production by the CNS.

We next performed bisulfite sequencing to provide insight into the CpG methylation pattern of the *Sox10* promoter region in normal and MO-treated dorsal neural tubes at stage 13. The results show that *Sox10* is highly methylated on the control side, and that 3B MO leads to an 18% reduction in the content of methylated CpGs on *Sox10*. In contrast, we found no differences in methylated CpGs between the 3B MO injected and uninjected sides on the *Snail2* promoter region (Fig. 6*B*). Accordingly, we propose a mechanism whereby *Sox10* acts as a "master regulator" whose shutdown is necessary for the termination of neural crest emigration. In turn, the up-regulation of *Sox10*, as a consequence of 3B MO treatment, induces the demethylation of other neural crest genes, such as *Snail2* and *FoxD3*, and their concomitant up-regulation.

Discussion

Long-term repression plays an important part in the programming of gene expression profiles in developing organisms. In this regard, de novo DNA methylation may be one of the main epigenetic marks used for cell fate restriction of progenitor cells during differentiation. The paralogs DNMT3A and 3B are both implicated in de novo DNA methylation, with some overlapping and other distinct functions. For example, we have shown that DNMT3A plays an important role during neural crest specification by acting as a molecular switch mediating the neural tube-to-neural crest fate transition (8). DNMT3A promotes neural crest specification by repressing, via promoter DNA methylation, neural genes like *Sox2* and *Sox3* in the neural crest territory, thus determining whether a cell becomes part of the CNS or PNS. In contrast to DNMT3A, DNMT3B lacks a function at this early stage; however, mutations in human DNMT3B result in ICF syndrome, in which patients exhibit facial abnormalities (13), suggesting a defect related to abnormal later neural crest development.

What regulates the spatial and temporal progression of the genetic program underlying development in such an exquisite

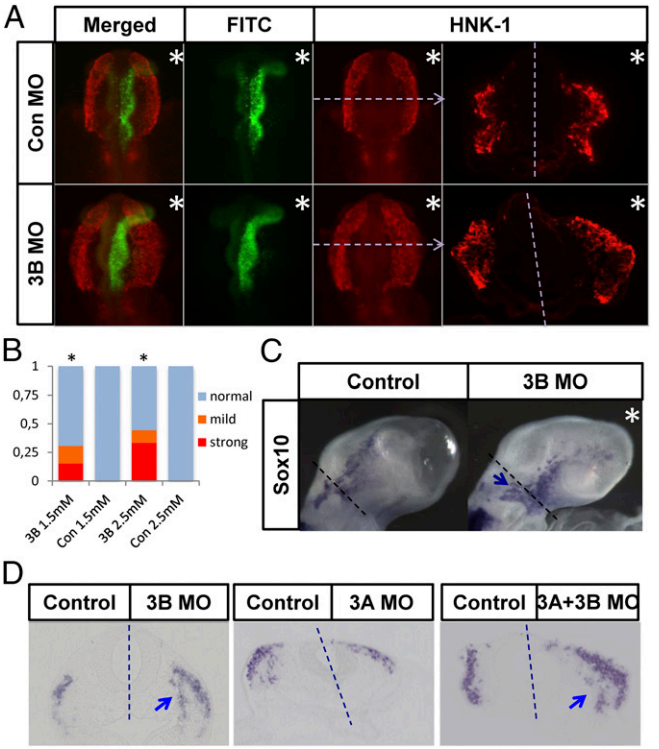

Fig. 4. DNMT3B knockdown results in extra migratory neural crest cells. (*A*) Embryos electroporated with 3B MO or Con MO were immunostained with anti-HNK1 antibody and sectioned. The 3B MO-injected sides of the embryos (asterisk) show increased numbers of HNK1-positive neural crest cells in both whole-mount and transverse section views. (*B*) Quantitation of the percentage of 3B MO-treated or Con MO-treated embryos with either a strong or mild phenotype of generating extra migrating neural crest cells on the electroporated side vs. the internal control side. $n = 13$ for 3B MO 1.5 mM; $n = 8$ for Con MO 1.5 mM; $n = 9$ for 3B MO 2.5 mM; $n = 8$ for Con MO 2.5 mM. $^*P < 0.05$, χ^2 contingency test. (*C*) Embryos collected at stage 13 continue to exhibit extra neural crest cells (arrow; $n = 5/7$) using *Sox10* as a marker on the 3B MO-treated side (asterisk) vs. the control side in both whole-mount and section views. (*D*) Embryos electroporated with DNMT3B MO, 3A MO, or 3A+3B MOs were assayed for *Sox10* expression by whole-mount in situ hybridization and sectioned. Whereas minor or no effect on the *Sox10* expression were observed for 3A MO alone, the double knockdown appeared similar to 3B MO knockdown, causing excess neural crest cell emigration (blue arrow).

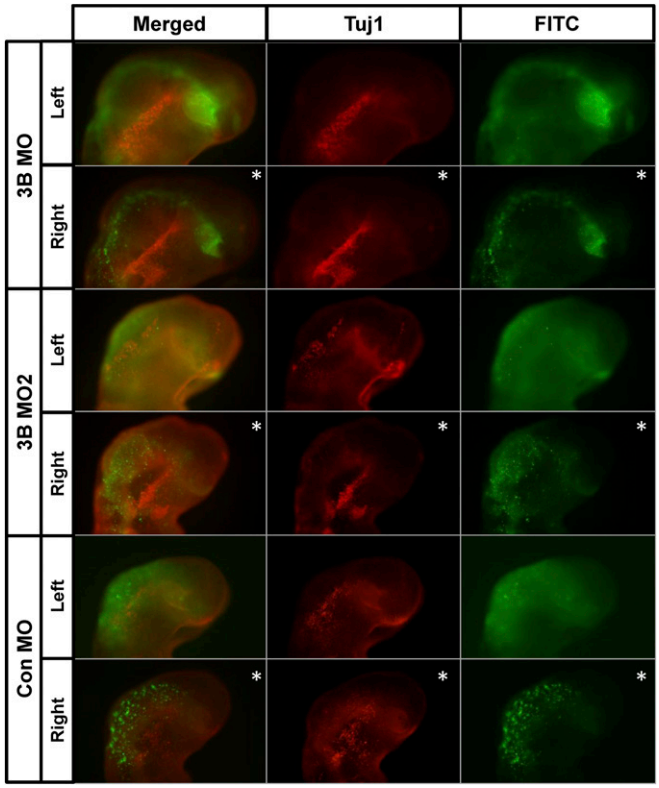

Fig. 5. DNMT3B knockdown results in premature differentiation of the trigeminal ganglia. The right half (asterisk) of each embryo was electroporated with 3B MO, 3B MO2, or Con MO (FITC). TUJ1 (red) served as a marker for neuronal differentiation in the forming trigeminal ganglia. Premature differentiation of the trigeminal ganglia (well-condensed ganglia with both ophthalmic and maxillo-mandibular lobes) was observed in the 3B MO-treated sides of the embryos compared with control sides, which showed normal ganglion aggregation. Four of six embryos treated with 3B MO, four of six embryos treated with 3B MO2, and none of six embryos treated with Con MO show premature differentiation of the trigeminal ganglia.

manner? Our results suggest that de novo DNA methylation, exerted by both DNMT3A and 3B, plays a dual role in the programs governing neural crest development. First, DNMT3A appears to limit the spatial boundary between neural crest versus neural tube progenitors, repressing the expression of neural markers in the neural crest forming territory. Second, DNMT3B appears to restrict the temporal window during which progenitors in the dorsal neural tube are competent to undergo EMT and produce neural crest cells. Thus, interfering with DNMT3A or 3B results in defects in PNS formation, but in separate temporal windows of neural crest development.

Our results demonstrate that de novo DNA methylation of the *Sox10* promoter region, exerted by DNMT3B, is required for cessation of cranial neural crest emigration. Interestingly, a recent paper (20) has shown that ectopic expression of Sox10 alone is sufficient to reprogram fibroblast cells in vitro to assume a neural crest identity. These authors further show that induction of these cells via Sox10 causes DNA demethylation of other neural crest genes, such as *FoxD3*, which are highly methylated in normal fibroblasts. Consistent with this, we show that the normal function of DNMT3B at the conclusion of cranial neural crest emigration is to shut down Sox10, a positive regulator of other neural crest genes like *FoxD3* and *Snail2*. Loss of DNMT3B during neural crest development in vivo thus results in abnormal maintenance of Sox10, which indirectly leads to up-regulation of the other neural crest markers and aberrant continuation of EMT.

A recent study in mice using neural crest-specific conditional deletion of DNMT3B failed to detect obvious defects in neural crest migration or differentiation into craniofacial and cardiac structures (21). One possible explanation for the difference from our results is that the neural crest defect in DNMT3B mutant mice may be related to a requirement for this protein before *Wnt1*-driven cre expression or in neighboring cell types. Alternatively, the difference may simply reflect species-specific differences, given that chicks lack DNMT3L, which is present in mice. Because DNMT3A and 3B are paralogs, it might not be surprising that each paralog has assumed slightly different roles in different species. DNMT3A and 3B are not redundant during neural crest development in chicks, but exhibit different and somewhat overlapping patterns in mice. Although DNMT3B null mice exhibit mostly normal neural crest development, Jacques-Fricke et al. (21) described ectopic migrating neural crest cells dispersed dorsal to the normal neural crest streams. This is similar to the prolonged emigration of neural crest cells that we observed in our 3B MO-treated chicken embryos, which results in abnormally migrating neural crest cells. This may be a less obvious phenotype in the null mice, considering that it is not possible to compare control and experimental sides in the same embryo as can be done in chicks.

In addition to de novo DNA methytransferases, other epigenetic regulators, including histone demethylases and histone

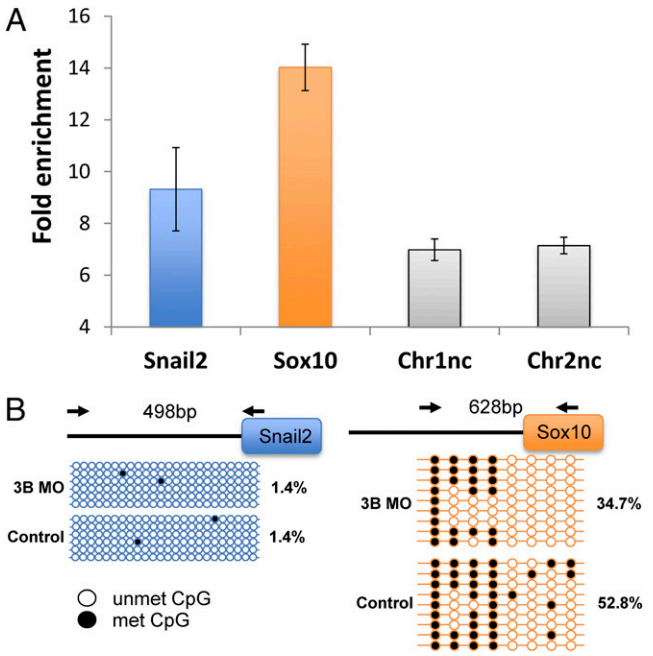

Fig. 6. DNMT3B binds directly to the promoter regions of *Sox10* and methylates CpGs to repress its expression. (*A*) Embryos were electroporated with a construct encoding exogenous DNMT3B with a FLAG tag on its N terminus. Micro ChIP was performed to detect the occupancy of FLAG-tagged DNMT3B on the promoter regions of *Sox10* and *Snail2*. The *y*-axis represents fold enrichment compared with IgG, and the *x*-axis represents the promoter regions of *Sox10*, *Snail2*, and two negative control regions that are in gene desert regions of chromosome 1 (Chr1nc) and chromosome 2 (Chr2nc). The results show high occupancy of DNMT3B-FLAG at the promoter of *Sox10* at stage 12, but no occupancy on the control regions and low levels for the *Snail2* promoter. Values in the graph are mean ± SD of a representative experiment. (*B*) Bisulfite sequencing profiles of CpG methylation on the *Sox10* and *Snail2* promoters from DNMT3B MO-treated and control dorsal neural tubes at stage 13. CpGs sequences are shown with filled (methylated) and open (unmethylated) circles. The numbers on the profiles represent the percentages of methylated CpGs.

deacetylases, also have been shown to play a crucial role in regulating the timing of neural crest specification or migration (22, 23). Histone demethylases, such as members of the Jumonji family, revert histone methylation (24). In neural crest development, JmjD2A (also known as KDM4A) regulates neural crest specification (18). *JmjD2A* is expressed in the neural crest territory during neural crest specification, and its loss of function causes dramatic down-regulation of neural crest genes such as *Sox10*, *Snail2*, and *FoxD3*. In vivo ChIP assays have revealed a direct interaction of JmjD2A with *Sox10* and *Snail2* promoter regions (18). JmjD2A is required for the demethylation of H3K9me3 at the promoter of *Sox10* and *Snail2* genes to allow neural crest specification. Moreover, a histone deacetylace (HDAC) repression complex plays an essential role in regulating neural crest migration. Premigratory neural crest cells from the dorsal neural tube undergo EMT to acquire migratory properties and travel to distant locations. The transcriptional repressor Snail2 has been reported to directly repress the adhesion molecule Cadherin6B in cranial premigratory neural crest cells (25–27). Epigenetic regulation plays a critical role in this repression (4). In particular, an interaction between PHD12, a member of the histone deacetylase complex, and SNAIL2 makes it possible to recruit the repressive complex SIN3A/HDAC to the *Cad6B* promoter region and, as a result, turn off *Cad6B* transcription via histone deacetylation to allow neural crest cells to gain migratory property (4). Taken together, this body of work demonstrates that epigenetic regulators influence many aspects of neural crest formation at different times and places.

Materials and Methods

In Ovo Electroporation. Embryos were electroporated at stage 4 or 8 as described previously (28), using DNMT3B morpholino (3B MO, over ATG codon: CGAGGCTCGTTACCATGCTCATCGC), DNMT3B morpholino 2 (3B MO2; upstream of ATG: GAACGGAGTGATGACAATGATACCT), or control morpholino (Con MO; CCTCTTACCTCAGTTACAATTTATA). DNMT3A MO (8) was used for some experiments. For rescue experiments, the construct included the coding region of DNMT3B-Flag in a pCI-IRES-H2BRFP vector. For each embryo, 1 mM MO + 0.5 μg/μL DNA was used for knockdown experiments, and 1 mM MO + 0.5 or 1 μg/μL DNA was used for rescue experiments. The embryos were then incubated until they reached the indicated stage of analysis.

In Situ Hybridization. Whole-mount chick in situ hybridization was performed as described previously (29).

Immunohistochemistry. For immunohistochemistry analyses, anti-TUJ1 (Covance) was diluted 1:250, and anti-HNK1 was diluted 1:10. Secondary antibodies against the primary antibody subtype were conjugated to Alexa Fluor 488, 568, or 350 dyes (Molecular Probes). Images were obtained with a Zeiss Axioskop2 microscope.

NanoString nCounter. Half-dorsal neural folds of 3B MO-treated embryos were dissected in lysis buffer (Ambion RNAqueous-Micro Isolation Kit) at stage 12. RNA lysates were hybridized to the probe set and incubated overnight at 65 °C, then washed and eluted following procedures detailed in the nCounter Prep Station Manual and counted with an nCounter Digital Analyzer.

Chromatin Immunoprecipitation. Embryos were electroporated with a DNMT3B-FLAG–containing vector. Dorsal neural tubes from embryos at stage 12 were dissected, cells were dissociated, cross-linked, and sonicated as described previously (18). Samples were evenly split among polyclonal anti-FLAG (Sigma-Aldrich), anti-IgG (Abcam), and input. Antibodies with protein A magnetic beads (Life Technologies) were incubated with the sonicated protein–DNA complex. Samples were washed, eluted, and reverse cross-linked. The final DNA pulldown was purified and served as a template for quantitative PCR.

Bisulfite Sequencing. Bisulfite treatment and sample recovery were carried out with the EpiTect Plus Bisulfite Conversion Kit (Qiagen) following the manufacturer's instructions. In brief, six dorsal neural tubes from control or 3B MO-treated sides were lysed and bisulfite-converted. The DNA thus-obtained was recovered in an EpiTect spin column. Two sets of nested primers were used to amplify by PCR the promoter regions of *Sox10* and *Snail2* genes from the bisulfite-converted DNA. The obtained product were gel-purified and cloned into the pGEM-T Easy Vector (Promega). Individual clones were sequenced.

Further experimental details are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Drs. T. Sauka-Spengler and M. Barembaum for helpful discussions. This work was supported by Grants F31DE021643 and 5 T32 GM07616 (to N.H.) and HD037105 and DE16459 (to M.E.B.).

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