Doublesex and mab-3–related transcription factor 5 promotes midbrain dopaminergic identity in pluripotent stem cells by enforcing a ventral-medial progenitor fate

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Understanding the control of cell-fate choices during embryonic stem cell (ESC) differentiation is crucial for harnessing strategies for efficient production of desired cell types for pharmaceutical drug screening and cell transplantation. Here we report the identification of the zinc finger-like doublesex and mab-3–related transcription factor 5 (Dmrt5) as a marker for mammalian ventral-medial mesencephalic neuroepithelium that give rise to dopamine neurons. Gain- and loss-of-function studies in ESC demonstrate that Dmrt5 is critically involved in the specification of ventral-medial neural progenitor cell fate and the subsequent generation of dopamine neurons expressing essential midbrain characteristics. Genome-wide analysis of Dmrt5-mediated transcriptome changes and expression profiling of ventral-medial and ventral-lateral mesencephalic neuroepithelium revealed suppressive and inductive regulatory roles for Dmrt5 in the transcription program associated with the ventral-medial neural progenitor fates. Together, these data identify Dmrt5 as an important player in ventral mesencephalic neural fate specification.

A major goal of embryonic stem cell (ESC) research is to direct the cells' differentiation toward specific cell types, especially those targeted by devastating degenerative diseases. The advent of induced pluripotent stem cell technology, with its promise for disease modeling, drug screening, and cell therapy, places further demand on a better understanding on the control of lineage/cell-fate specification from pluripotent stem cells. One neuronal cell type in particular, the midbrain dopaminergic (mDA) neuron, is a prime target in applied stem cell research because of its association with Parkinson's disease.

The mDA neurons are generated in the floor plate (FP) region of the ventral midbrain and are uniquely identified by their coexpression of tyrosine hydroxylase (TH) with the mDA-specific homeobox protein Pitx3 (1, 2). During development, local inductive signals—Shh, FGF8, and Wnt1—induce distinct cell-fate potentials through initiation of transcriptional cascades that govern the subsequent differentiation, migration, and maturation of the ventral-most progenitors into mDA neurons (3–5). The distinct cell-fate potentials of ventral midbrain progenitors are defined by domain-identifiable expression of transcription factors. For example, the $Lmx1a^+Foxa2^+$ FP exclusively gives rise to mDA neurons, whereas the ventral-lateral domains marked by Meis2, Mab21l2, Helt, and Lhx1 produce glutamatergic or GABAergic neurons (6–9). Perturbation of such a transcription "code" seen in genetic studies often led to misspecification of progenitor identity and subsequently to neural transmitter phenotypes (10–12). These studies demonstrate the mechanism of cell-fate determination to be a balance of the activation of "specification" programs and the repression of alternative fates, as observed in the spinal cord (13). However, how transcription factors coordinate distinct fate choice in the ventral midbrain remains poorly understood.

The *Drosophila doublesex* and *Caenorhabditis elegans mab-3* genes encode transcription factors that are characterized by a conserved zinc finger-like DNA-binding motif known as the DM domain. Several vertebrate homologs have been identified and termed doublesex and mab-3–related transcription factors 1–8 (Dmrt1 to Dmrt8) (14). The most conserved and well-studied function of Dmrt proteins is in sexual development. However, some *Dmrt* genes are expressed and function in nongonadal tissues. For example, *Xenopus Dmrt4* is expressed in anterior neural tissues and is required for neurogenesis in the olfactory system (15). Here we report that *Dmrt5* is a gene marker of mammalian ventral-medial mesencephalic neural progenitors. Using conditional overexpression and shRNA knockdown–based ESC models, we validated Dmrt5 as a transcription regulator controlling ventral-medial mesencephalic neuronal progenitor and mDA neuronal-fate choice.

Results

Restricted Expression of *Dmrt5* in the Developing Ventral-Medial Mesencephalon. We identified *Dmrt5* from a differential microarray expression screen of ventral-medial midbrain cell populations. Whole-mount in situ hybridization revealed precise spatial and temporal regulation of *Dmrt5* expression in both mouse and chicken embryos. In the ventral midbrain, *Dmrt5* is first expressed at the midbrain/forebrain border at embryonic day 9.5 (E9.5) in mice and Hamilton and Hamburger stage 20 (st.20) in chickens. Expression extends posteriorly to encompass the entire medial ventral midbrain (Fig. 1 *A* and *C*). Outside the ventral midbrain, *Dmrt5* expression was found in the dorsal telencephalon as well as in the optic stalk and early olfactory bulb. Combining *Dmrt5* in situ hybridization with antibody staining for regional markers of the ventral-medial and lateral midbrain neuroepithelium, we found that the $Dmrt5^+$ region contains the $Lmx1a^+$ cell population (Fig. 1*A*). Like Foxa2 and Lmx1a, $Dmrt5^+$ cells occupy almost the entire thickness of the neural epithelium at E10.5 (6, 12). The lateral boundaries of *Dmrt5* expression lie immediately ventral to $Nkx2.2^+$ neuroepithelium (Fig. 1*A*), forming two mutually exclusive expression domains. At E12.5, *Dmrt5* expression was found primarily in the ventricular zone of the FP and adjacent ventral midbrain (Fig. 1*B*). With the exception of a small number of cells in the most medial intermediate zone that coexpress *Dmrt5* and TH, the vast majority of *Dmrt5*-stained cells lay dorsal to the differentiated TH^+ dopamine neurons in the marginal layer (Fig. 1*D*). Together, these expression studies indentified Dmrt5 as a marker for mesencephalic dopamine neural progenitors.

Dmrt5 Regulates Ventral Mesencephalic Gene Expression in Naïve Neuroepithelium. Given this highly specific expression profile, we used the chick electroporation system to examine the patterning effects of *Dmrt5* in vivo. A mouse *Dmrt5* expression plasmid was electroporated into naïve chick midbrain neuroepithelium at st.10. The embryos were allowed to develop for 24 h until st.17 and

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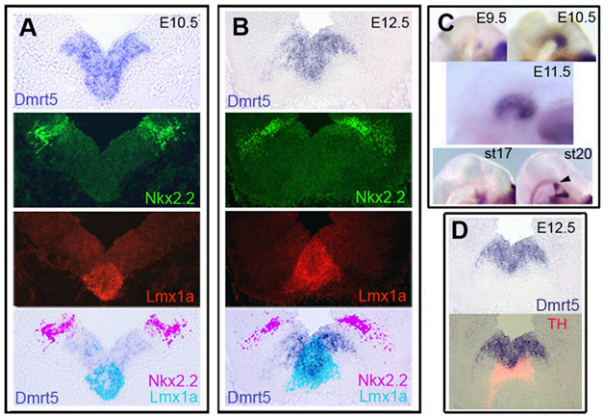


Fig. 1. Dmrt5 expression in the ventral midbrain. (*A*) Coronal section of an E10.5 mouse ventral midbrain showing ventral-medial expression of *Dmrt5*. (*B*) Coronal section of an E12.5 ventral midbrain. *Dmrt5* expression is primarily restricted to the ventricular zone. (*C*) Lateral views of E9.5, E10.5, and E11.5 mouse midbrains (*Top* and *Middle*) showing the caudally extending *Dmrt5* expression and chick midbrains (*Bottom*) just before (st17) and after (st20) the onset of *Dmrt5* expression (black arrowhead). (*D*) Coronal section of an E12.5 ventral midbrain showing slight overlap of *Dmrt5* and TH expression.

were analyzed for expression of *Foxa2*, a medial midbrain marker, and *Mab21l2*, a gene shown to maintain dorsal identities in early neural development (8). Both genes are highly conserved between the mouse and chick and show a nonoverlapping, spatially restricted expression pattern in the developing midbrain (8, 12).

As can be seen in the nonelectroporated side of the midbrain, *Mab21L2* is expressed in the dorsal midbrain and extends ventrally to end in a ventral-lateral position (Fig. 2*A*). This expression was abolished in the *Dmrt5* electroporated neuroepithelium in all embryos (4/4). In contrast, *Foxa2* expression was ectopically induced in ventral-lateral cells by Dmrt5 (4/5) (Fig. 2*B*). No alteration in normal gene expression was seen in nonelectroporated, contralateral side of the embryo (Fig. 2). Our data provide proof that Dmrt5 is capable of regulating ventral-lateral and medial patterning genes in vivo. The ability of Dmrt5 to override endogenous gene induction pathways of these patterning genes points to a direct regulatory role for Dmrt5 in specification of the ventral-medial midbrain cell fate.

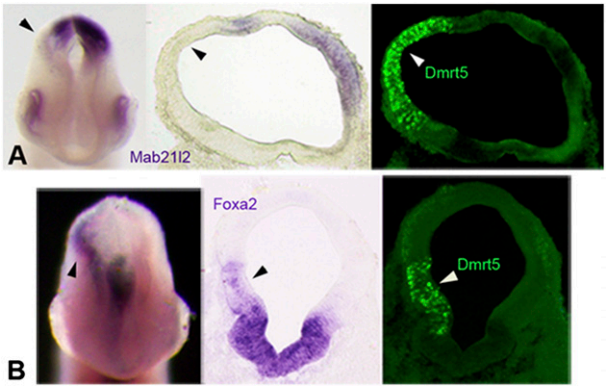


Fig. 2. Dmrt5 regulates regionally specific mesencephalic gene expression in the chicken embryo. Naïve chick midbrain neuroepithelium was electroporated with a mouse Dmrt5 expressing plasmid and hybridized for chick *Mab21l2* or *Foxa2* at st.17. (*A*) Frontal view (*Left*) and coronal midbrain section (*Center*) illustrating the loss of *Mab21l2* expression (black arrowheads) in ectopic *Dmrt5*-expressing (*Right*) tissue (white arrowhead). (*B*) Frontal view (*Left*) and coronal midbrain section (*Center*) illustrating the induction of *Foxa2* expression (black arrowheads) in ectopic Dmrt5-expressing (*Right*) tissue (white arrowhead).

Dmrt5 Promotes mDA Neural Progenitor Marker Expression in Neuralized ESCs. Examination of intrinsic transcriptional regulators and extrinsic niche factors critical for mDA development in ESC differentiation has proved to be a powerful means for illuminating or validating their functions in dopaminergic neuron fate specification (6, 16–20). Notably, engineered expression of mDA transcription factors such as *Lmx1a* and *Pitx3* induced midbrain regional character in derived dopamine neurons (6, 19). Overexpression of Nurr1 increased the number of TH^+ neurons but without apparent impact on midbrain regional identity (18, 21). To explore the specification potential of Dmrt5 in greater detail, we generated a number of tetracycline-inducible *Dmrt5* transgenic mouse ESC lines using ESCs, which harbour the reverse tetracycline-controlled transactivator (rtTA ESCs). (Fig. S1). After verifying the ability of Dmrt5-ESCs to produce significant levels of Dmrt5 protein, we selected two lines to investigate the effects of *Dmrt5* expression on dopamine neuron differentiation after a monolayer-based neural differentiation method (22, 23).

Dmrt5 expression in the ventral mesencephalon is restricted to mitotic neuroepithelial cells and precedes the onset of dopaminergic neurogenesis. Thus, it is likely to function at neural progenitor stage. We therefore induced the Dmrt5 transgene at the peak of neural progenitor production and determined the effect of Dmrt5 induction on ventral-medial midbrain neural progenitor marker expression by quantitative PCR (qPCR) from day 6 to day 12 monolayer differentiation (d6–12 MD). We found that the induction of Dmrt5 did not affect pan-neuroepithelial marker gene level but resulted in a four- to fivefold increase in transcript levels of *Foxa2*, *Lmx1a*, and *Msx1*, three transcription factors required for the specification of ventral-medial cell identities and normal dopamine neuronal development (Fig. 3*A* and Fig. S2*A*). The upregulation of these marker genes was detected concurrent with the

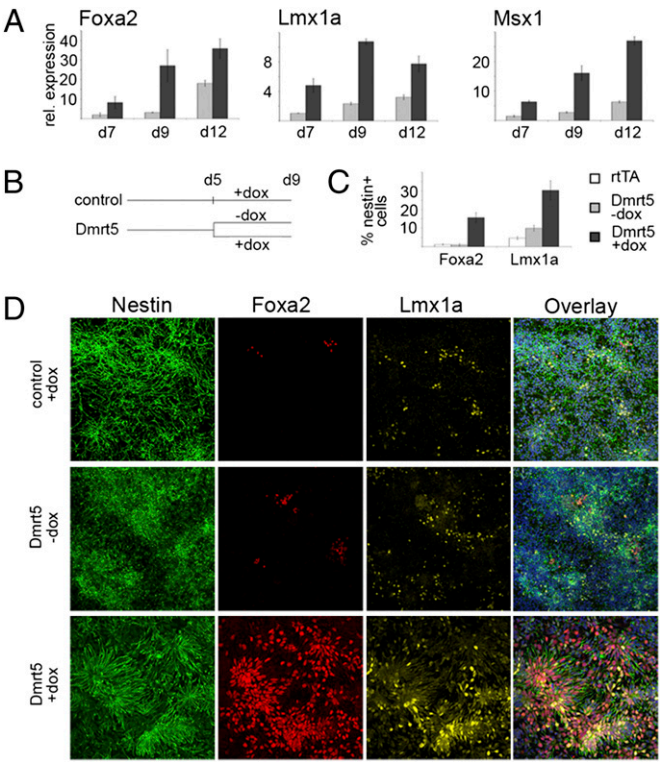


Fig. 3. Dmrt5 induces ventral-medial midbrain progenitor phenotype. (*A*) qPCR analysis for mDA regulators. *Dmrt5* overexpression was induced from d5 to d12 MD. (*B*) Schematics of the experimental program for immunostaining in *C* and *D*. (*C*) Quantitative data of $Nestin^+$ cells expressing Foxa2 or Lmx1a in d9 MD. (*D*) Immunostaining of d9 MD derived from rtTA control and Dmrt5 ESCs. Data in *A* and *C* are presented as mean ± SEM of three independent replicates.

induced transgenic Dmrt5 at d7 MD, and they remained at a higher level than in control cultures at all time points analyzed.

We then examined the expression at the protein level of two key mDA markers, Foxa2 and Lmx1a, by immunocytochemical staining (Fig. 3*B*). After 4 d of doxycycline treatment (i.e., at d9 MD) more than 30% of Nestin$^+$ neural progenitors expressed Lmx1a (30.3 ± 5%) and 15.6 ± 2.6% became Foxa2$^+$ in the Dmrt5 cultures. In contrast, only a small proportion of Nestin$^+$ cells in the doxycycline-treated rtTA control cultures became Lmx1a$^+$ (4.6 ± 0.8%) or Foxa2$^+$ (1.2 ± 0.4%) (Fig. 3 *C* and *D*). Similar numbers of Foxa2$^+$ neural progenitor cells were obtained in Dmrt5-ESC and rtTA cultures not treated with doxycycline, although we detected an increased number of Lmx1a$^+$ cells in noninduced Dmrt5-ESC cultures; the latter could be attributable to the leakage of *Dmrt5* transgene expression in the absence of doxycycline (Fig. S1*D*). Our data suggest that Dmrt5 is capable of promoting the Lmx1a$^+$ and Foxa2$^+$ neural progenitor phenotype.

Dmrt5 Restricts Shh-Induced Neural Progenitors to Ventral-Medial Identity by Suppressing Ventral-Lateral Neuroepithelial Characteristics. Dmrt family proteins have been shown to function as transcription repressors as well as activators (14). The mutually exclusive expression between *Dmrt5* and Nkx2.2 in the midbrain raised the possibility that Dmrt5 inhibits ESC-derived neural progenitors from adopting alternative cell fates, such as those associated with the ventral-lateral midbrain. To test this hypothesis, we examined the effect of Dmrt5 on the expression of nondopaminergic ventral-lateral marker genes, including *Nkx2.2*, *Isl1*, *Lhx1*, *Helt*, *Brn3a*, *Meis2*, and *Mab21l2* (7–9, 24). By qPCR, we found that Dmrt5 overexpression resulted in a striking down-regulation of all these marker genes (Fig. 4*A* and Fig. S2 *B* and *C*).

A number of ventral-laterally expressed transcription factors, such as Nkx2.2 and Isl1, are induced by Shh (13). Therefore, Dmrt5-mediated suppression might involve Shh signaling. We repeated the above experiments in cultures treated with Shh or cyclopamine, an inhibitor of Shh signaling. Shh added to the cultures for 2 d (d3–5 MD) or 6 d (d3–9 MD) induced an increase in the transcript levels of *Nkx2.2* and *Isl1* in ESC-derived neural cultures, as Shh signaling does in the developing neuroepithelium (13). The presence of cyclopamine reduced their expression (Fig. 4*A*). However, conditional *Dmrt5* transgene expression in the presence of Shh or cyclopamine still was able to cause a prominent repression of the two genes (Fig. 4*A*).

A regulatory role for Shh on *Lhx1*, *Helt*, *Brn3a*, and *Meis2* expression has not been previously reported. Unlike the induction of *Nkx2.2* and *Isl1*, we found that Shh treatment results in a marked inhibition in the expression levels of *Lhx1*, *Helt*, *Brn3a*, and *Meis2* (Fig. 4*A*), which are further reduced by the overexpression of Dmrt5. Cyclopamine treatment had no significant effect on the levels of *Lhx1*, *Helt*, *Brnl3a*, and *Meis2*, but Dmrt5 continued to reduce the expression of these genes under this condition. Foxa2 has been suggested to suppress Helt and Lhx1 expression in the midbrain (12); however, the barely detectible levels of Foxa2 in the presence of cyclopamine make a Foxa2 role in the observed decrease of Helt and Lhx1 unlikely (Fig. S3). Together, these results indicate that, independently, Dmrt5 negatively regulates the ventral-lateral midbrain gene-expression profile irrespective of its responsiveness to Shh.

To ask whether the observed transcript changes correlates with protein expression, we performed immunocytochemical staining for Nkx2.2, Isl1, and Lhx1, for which reliable commercial antibodies are available. In standard MD cultures, Nkx2.2$^+$ and Isl1$^+$ cells were present in small numbers, whereas Lhx1$^+$ cells were readily detectable in most microscopic fields. Dmrt5 overexpression greatly reduced the production of Lhx1$^+$ cells, whereas Nkx2.2$^+$ and Isl1$^+$ cells became almost nondetectable. To obtain appreciable numbers of Nkx2.2$^+$, Isl1$^+$, and Lhx1$^+$ cells in the Dmrt5-overexpressing condition, and thus a reliable quantitative analysis, we used a 6-d Shh treatment from d3 to d9 MD. Again, we observed a clear reduction in the number of Nkx2.2$^+$, Isl1$^+$, and Lhx1$^+$ cells in Dmrt5-overexpressing cultures compared with the no-doxycycline controls (Fig. 4*B*).

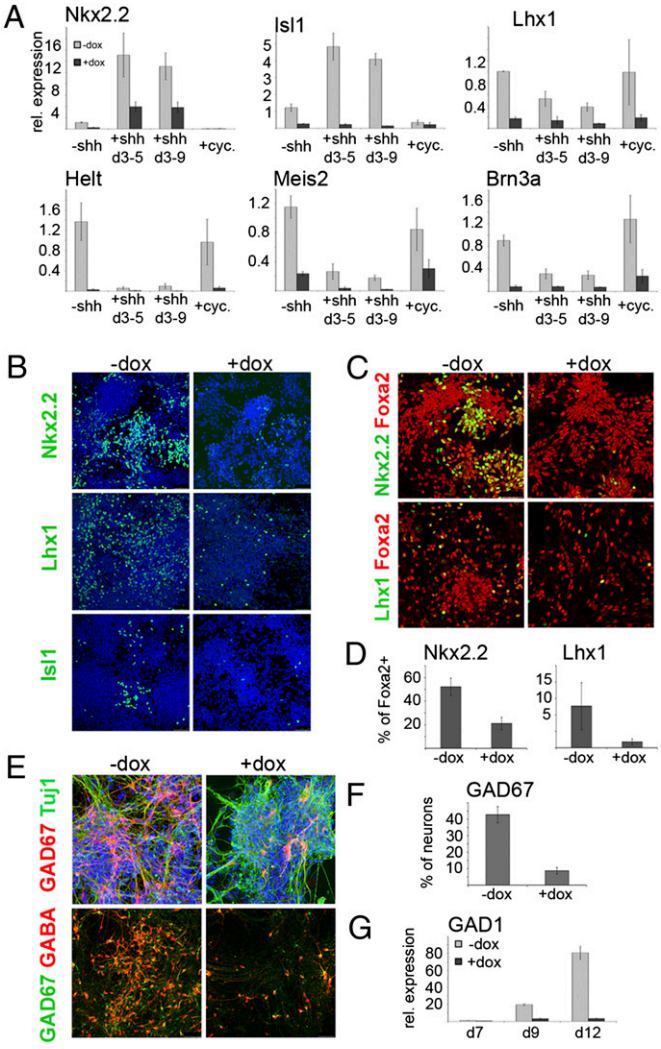


Fig. 4. Dmrt5 inhibits the ventral-lateral mesencephalic profile. (*A*) Monolayer cultures of Dmrt5-ESC with or without doxycycline from d5 either received no further treatment or were treated with Shh from d3–5 (+shh d3-5) or from d3–9 (+shh d3-9) or were treated with cyclopamine from d3. At d9, the cultures were analyzed by qPCR for ventral-lateral genes. (*B* and *C*) Dmrt5-ESCs were differentiated in the presence of Shh from d3 and treated with doxycycline or vehicle from d5. Cultures were stained at d9 with antibodies against Nkx2.2, Islet1, or Lhx1 (*B*) or Foxa2/Nkx2.2 or Foxa2/Lhx1 (*C*). (*D*) Quantitation of *C* showing the reduction of Nkx2.2$^+$ and Lhx1$^+$ cells within the Foxa2 population in Dmrt5 cultures. (*E*) d14 MD stained for GABAergic neuronal markers GAD67 and GABA. (*F*) Quantitation of *E* showing the reduction of GABAergic neurons. (*G*) qPCR analysis for *Gad1*.

We found that a significant proportion of neural progenitors in Shh-treated cultures are Foxa2$^+$Nkx2.2$^+$ and Foxa2$^+$Lhx1$^+$ (Fig. 4*C*). However, expression of Dmrt5 resulted in a 60% and 75% reduction, respectively, of Foxa2$^+$ cells coexpressing Nkx2.2 (from 52.2 ± 7.5% to 21.2 ± 5.2%) and Lhx1(from 17.2 ± 6.4% to 4.2 ± 2.7%) (Fig. 4 *C* and *D*), exposing a role for Dmrt5 in fine-tuning the ventral-medial identity. Consistent with the suppression of the nondopaminergic progenitor profile, we observed a reduction in the number of GABAergic neurons as demonstrated by GABA and GAD67 antibody staining, which was preceded by decreased transcript levels of the GABAergic regional and subtype specification genes, *Helt* and *Gad1* (Fig. 4 *E*–*G*).

The observed decrease and increase of ventral-lateral and ventral-medial phenotype is unlikely caused by toxicity of doxycycline because it had no effect on the number of Pax6$^+$ and Nestin$^+$ neural progenitors or RNA expression levels for *Foxa2*,

Lmx1a, or *Nkx2.2* in the rtTA control cultures (Fig. S4). Furthermore, we observed no increase in progenitor apoptosis or selective expansion of Lmx1a$^+$ subpopulation as determined by activated caspase3 staining and double immunostaining for Ki67 and Lmx1a (Fig. S5). Collectively, our data suggest that Dmrt5 promotes ventral-medial mesencephalic fate choice by suppressing transcription programs associated with alternative cell fates.

Dmrt5 Deficiency Compromises ESC Differentiation Toward Ventral-Medial Cell Fate. To investigate a Dmrt5 requirement in mDA progenitor fate choice, we generated shRNA-based conditional *Dmrt5* knockdown ESC lines by using a pSico-based vector (25) (Fig. S6*A*). The parental ESCs (R26) constitutively express CreERT2, a Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ERT2), under the control of the constitutive and ubiquitous Rosa26 promoter. Thus, in the presence of tamoxifen, the puromycin-resistance cassette that functions as a transcription stop will be excised, leading to the (nonreversible) expression of *Dmrt5* shRNA. Two independent ESC lines, C859 and C1021, which carry distinct shRNA target sequences, consistently showed 40–60% reduction of the *Dmrt5* transcript in ESC-derived neural cultures when Cre activity was induced by tamoxifen in undifferentiated ESCs or during differentiation consistently (Fig. 5*A* and Fig. S6).

The noninduced and induced C859 and C1021 (referred to hereafter as *Dmrt5*KD) showed comparable levels of *Sox1* and *Nestin* transcripts as well as the numbers of Nestin$^+$ cells at d7 and d9 MD (Fig. 5 *A*–*C* and Fig. S6), suggesting that the lack of *Dmrt5* does not affect the neuroectoderm lineage commitment of ESCs. However, we found that *Dmrt5*KD resulted in a more than twofold reduction in the levels of *Foxa2* and *Msx1* and a fivefold decrease of *Lmx1a* at d7 and d9 MD (Fig. 5*A*). This effect was specific to *Dmrt5* knockdown because tamoxifen treatment on the parental R26 ESCs did not affect the levels of Foxa2, Lmx1a, or other neural markers tested (Fig. S6). Conversely, we observed an increased level of *Helt*, *Meis2*, *Lhx1*, and *Isl1* in *Dmrt5*KD neural cultures compared with the noninduced controls (Fig. 5*A*). The above finding was further supported by a significant reduction in the number of Foxa2$^+$ (60%) and Lmx1a$^+$ (80%) neural progenitors in *Dmrt5* KD cultures treated with Shh (Fig. 5 *B* and *C*). Taken as a whole, our data reveal a requirement for Dmrt5 in ventral midbrain neural progenitor fate specification.

Genome-Wide Analysis Supports a Role for Dmrt5 in Regulating Ventral Mesencephalic Neuroepithelial Cell Fates. Further insight into the functionality of Dmrt5 and transcription networks potentially regulated by Dmrt5 is derived from Affymetrix expression profiling using neural progenitors derived from the Dmrt5-overexpressing and parental control ESCs with or without doxycycline treatment (Fig. 6*A*). Results showed 987 entities with qualified fold change > 2 and $P < 0.05$ by one-way ANOVA. Of these, 485 were down-regulated and 502 were up-regulated by Dmrt5 (Fig. 6*B* and Dataset S1). These changes are specific to Dmrt5 because the effect of doxycycline on parental rtTA cell gene expression was negligible.

We were particularly interested in investigating whether Dmrt5-responsive genes correlate with Dmrt5 region-specific gene expression in the developing midbrain. To this end, we performed another microarray analysis to identify genes differentially expressed in the mesencephalic FP and the adjacent, nonoverlapping ventral-lateral midbrain region (VL) of 10.5-d postcoitum (dpc) mouse embryos (Fig. 6*C*). Using a cutoff of fold change > 2 and $P < 0.05$, we identified 653 FP-enriched entities and 451 VL-specific entities (Dataset S1). The FP- and

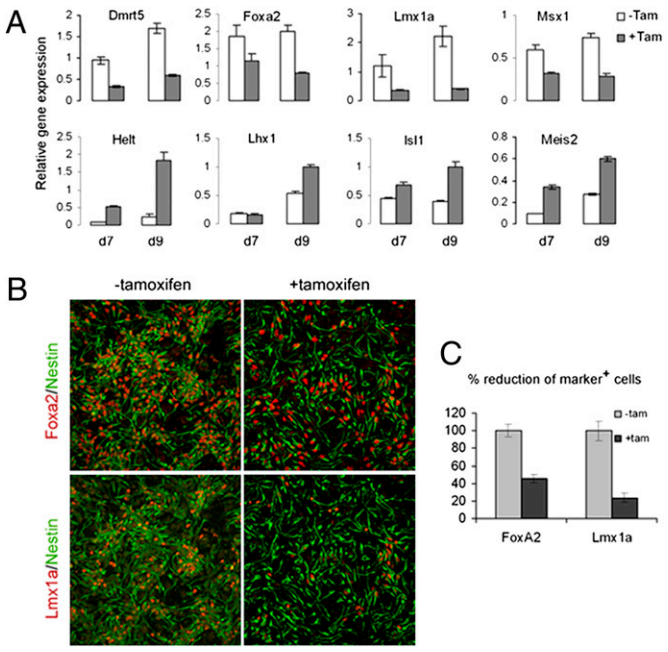
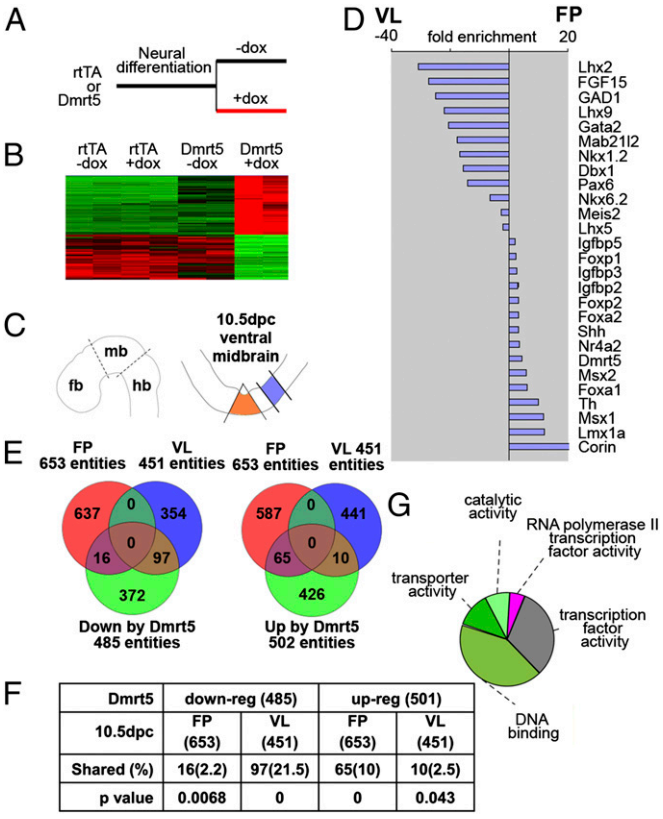


Fig. 5. Dmrt5 knockdown compromises ESC differentiation toward a ventral-medial cell fate. (*A*) qPCR analysis of d7 and d9 MD of the C859 Dmrt5 shRNA ESCs. *Dmrt5*KD was induced by tamoxifen for one passage before MD. (*B*) Immunostaining for Foxa2 and Lmx1a at d9 MD of C859. To obtain reliable numbers of Foxa1$^+$ and Lmx1a$^+$ cells, Shh was applied from d3 to d5 MD. (*C*) Quantitation of *B* showing percentage reduction of Foxa1$^+$ and Lmx1a$^+$ within Nestin$^+$ neural progenitor population.



Dmrt5	down-reg (485)		up-reg (501)	
10.5dpc	FP (653)	VL (451)	FP (653)	VL (451)
Shared (%)	16(2.2)	97(21.5)	65(10)	10(2.5)
p value	0.0068	0	0	0.043

Fig. 6. Genome-wide analysis for Dmrt5-mediated gene regulation. (*A*) Schematics of experimental design for microarray analysis of ESC-derived neural progenitors using the rtTA-parental and Dmrt5-5 cells. (*B*) Heat map showing Dmrt5-regulated gene expression. (*C*) Schematics of E10.5 dissection for isolating midbrain FP and ventral-lateral region (VL) neural tissues. (*D*) Examples of FP and VL differentially expressed genes identified by the microarray. (*E*) Venn diagram highlighting the overlap of Dmrt5-regulated genes and those preferentially expressed in the FP or VL. (*F*) Summary and statistical analysis of shared genes within groups indicated. (*G*) Gene ontology analysis of the Dmrt5 down-regulated/VL genes.

VL-specific entity lists contain all genes known to be differentially expressed in the respective regions of midgestation mouse midbrain (Fig. 6*D*), demonstrating the high quality of this dataset. Cross-comparison between the Dmrt5 up- and down-regulated lists with the FP- and VL-specific entities revealed remarkably parallel enrichments of the Dmrt5-regulated genes. The Dmrt5 down-regulated list matched significantly more entities in the VL list than in the FP list (97 vs. 16 entities or 21.5% vs. 2.2% of all FP or VL entities, respectively) (Fig. 6 *E* and *F*). Interestingly, the Dmrt5 down-regulated/VL-specific list is rich in transcription factors important for lateral and dorsal midbrain patterning, such as Pax3, -6, and -7 and Lhx9 (Fig. 6*G* and Dataset S2), as well as those already shown with the candidate gene approach to be suppressed by Dmrt5 (Fig. 4). Conversely, Dmrt5 up-regulated entities matched more of the FP list than those in the VL list (65 vs. 10 entities or 10% vs. 2.5%) (Fig. 6 *E* and *F*). Hypergeometric distribution analysis confirmed the strongest significance for the Dmrt5 down-regulated/VL and Dmrt5 up-regulated/FP entities (*P* value was almost 0) (Fig. 6*F*). Thus, our global gene-expression analysis provides strong independent support that Dmrt5 promotes ventral-medial midbrain neuroepithelial fate.

Dmrt5 Enhances the Generation of DA Neurons Exhibiting Key Midbrain Complements. We next determined the effect of Dmrt5 on postmitotic dopamine neuron production with particular attention on their midbrain regional marker expression. To temporally mimic neural progenitor expression of endogenous *Dmrt5*, we induced *Dmrt5* transgene expression for 4 d during the peak of neural progenitor production between d5 and d9 MD. We omitted exogenous dopamine-inducing factors to reveal Dmrt5 activity with minimum input from other signals. As previously reported (6, 23), few TH^+ neurons in the parental or noninduced control cultures expressed Pitx3, Lmx1a, or Foxa2 (Fig. 7 *A* and *B*). The majority of TH^+ cells in these cultures were dispersed and had an immature neuronal morphology as demonstrated by fewer and shorter processes. In contrast, a significant proportion of TH^+ neurons in doxycycline-induced Dmrt5 cultures coexpressed Pitx3, Lmx1a, and Foxa2 (Fig. 7*A*): 40% of TH^+ neurons were $Pitx3^+$, 37% were $Lmx1a^+$, and 59% were $Foxa2^+$ (Fig. 7*B*). Additionally, most of the TH^+ cells were found in large clusters and had a mature neuronal morphology with long and elaborate processes and small cell bodies (Fig. 7*A*). Furthermore, Dmrt5 induction led to a 63 ± 15% increase in the total number of TH^+ neurons compared with the parental control cultures, leading, significantly, to an increase in the total number of TH^+ cells coexpressing Pitx3, Lmx1a, and Foxa2 (Fig. 7*C*). Interestingly, although the vast majority of TH^+ neurons coexpressed an A9 (substantia nigra) DA marker, Girk2, only a small proportion of TH^+ cells expressed calbindin, which is preferentially expressed in the A10 (ventral tegmental area) subpopulation (Fig. 7*D*). Dmrt5 induction had little impact on coexpression of TH with Nurr1 (Fig. 7 *A* and *B*). This observation is not surprising because Nurr1 controls neural transmitter phenotype by regulating *Th* gene expression, and this activity does not appear to be coupled to regional or even neuronal phenotype (18). Therefore, our data demonstrate that Dmrt5 is capable of inducing mDA neuronal character in ESCs. This activity distinguishes Dmrt5 from Lmx1a, which requires cooperation with Shh to promote mDA neuronal phenotype.

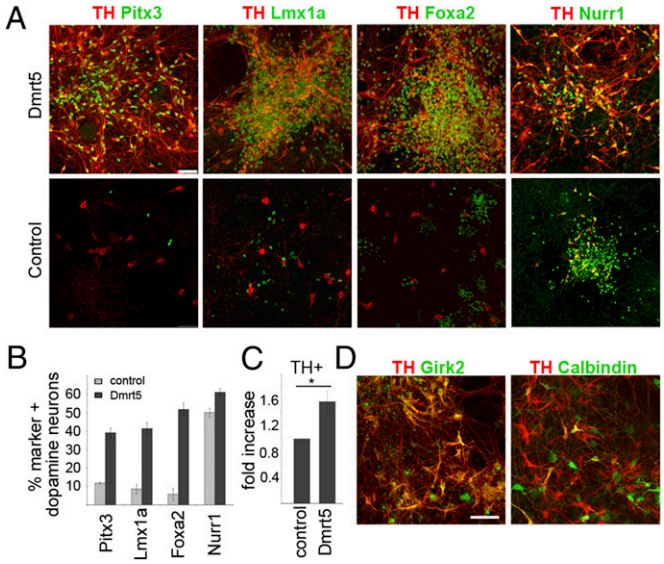


Fig. 7. Dmrt5 overexpression promotes the generation of DA neurons exhibiting midbrain characteristics. (*A*) d16 MD of the parental rtTA and Dmrt5-ESCs were double-stained for TH (green) and mDA neuronal markers Pitx3, Lmx1a, Foxa2, or Nurr1 (red). (*B*) Percentage of TH^+ neurons coexpressing mDA markers. (*C*) Quantification of the number of TH^+ neurons. The numbers are normalized to the parental control culture in each independent experiment ($n = 3$). *$P < 0.01$, Student's *t* test. (*D*) d16 Dmrt5 cultures were double-stained for TH (red) and Girk2 (green) or TH (red) and calbindin (green). (Scale bars: 50 μm.)

Discussion

In this study, we identified an important role for the zinc finger–containing transcription factor Dmrt5 in mDA neuroepithelial cell-fate determination. We show that Dmrt5 enhances the acquisition of mDA neuronal fate at the expense of GABAergic neurons, and its deficiency compromises the production of mDA subtype. This effect is achieved by suppressing the transcriptional program controlling the ventral-lateral neuroepithelial identity while promoting ventral-medial midbrain neural progenitor fate. By using a combinatorial approach involving conditional gain and loss of function of Dmrt5 during neural differentiation of ESCs, analysis of global gene expression of ESC-derived neural progenitors and embryo-derived neural tissues, as well as the ectopic expression of mouse Dmrt5 in naïve chick neuroepithelium, we provide clear evidence that Dmrt5 promotes specification of the dopamine progenitor population.

Over the last decade, the roles of a number of genes in the differentiation of ventral midbrain DA neurons have been highlighted. The early expression of Shh is required to potentiate ventral cell fates (12, 26), and Shh signaling, in combination with Otx2 expression, locates the anterior-ventral neuronal progenitor domains such as the FP and, more laterally, those marked by Nkx2.2 expression (10, 26). These domains are established in early neurogenesis, followed by the differentiation of domain-specific neural subtypes; the maintenance of these domains is a delicately balanced interaction between transcriptional cascades.

Dmrt5 overexpression led to dramatic suppression of Nkx2.2, which is consistent with the absence of any overlap in the juxtaposed Dmrt5 and Nkx2.2 expression domains. Conditional loss of Otx2 results in ectopic expression of Nkx2.2 in the ventral-medial mesencephalon and, subsequently, loss of dopaminergic neurons and induction of rostral serotonergic neurons (27). Nkx2.2 deletion in the Otx2 mutant background rescues the mDA deficiency, indicating that suppression of Nkx2.2 in the ventral-medial midbrain is necessary to permit dopaminergic cell-fate specification. Dmrt5 overexpression did not induce Otx2; thus, the observed suppression of Nkx2.2 by Dmrt5 is unlikely to be via Otx2. Foxa2 has also been implicated in the regulation of Nkx2.2. Loss of Foxa1/2 results in ectopic Nkx2.2 expression in the ventral-medial midbrain. Foxa2 can occupy the *Nkx2.2* promoter (12), suggesting a direct repressive regulation. The mechanism of Nkx2.2 regulation by Foxa2, however, is not clear and may be context-dependent because ectopic medial expression of $Nkx2.2^+$ domain was also observed in the Otx2 conditional knockout, despite the medial maintenance and lateral expansion of Foxa2 expression in these mice (10, 12). Thus, the maintenance of the Nkx2.2 expression domains by Otx2 is via a pathway independent of that of the Foxa2 restriction on the Nkx2.2 expression domain in the ventral midbrain. Given that Foxa2 coexpresses with Nkx2.2 in

a population of hindbrain cells (28), it is conceivable that Foxa2 regulates Nkx2.2 cooperatively with a region-specific partner.

This study revealed a robust repressive role for Dmrt5 on several other ventral-lateral genes' expression independent of FP/Shh activity, as exemplified by the finding that Dmrt5 strongly antagonized the induction of Nkx2.2 by Shh and completely blocked its ability to induce Isl1 (Fig. 4). Analysis of the Foxa1/2 compound-mutant mice revealed ectopic expression of *Helt* and *Lhx1* in the Foxa1/2-deficient FP, implicating a repressor role of Foxa1/2 (12). Our observation indicates that Dmrt5 is able to regulate *Helt* and *Lhx1* expression independent of Foxa1/2 because robust reduction of *Helt* and *Lhx1* by Dmrt5 was not affected when Foxa2 level was either elevated (by Dmrt5) or negligible in cyclopamine-treated cultures.

Genetic studies of mesencephalic FP genes demonstrate that domain identity is also highly interactive. Lmx1a is capable of inducing ectopic mDA differentiation (6) but only in collaboration with Foxa2 (11, 12). Our work here shows that Dmrt5 promotes a $Msx1^+$, $Lmx1a^+$, and $Foxa2^+$ mDA progenitor phenotype, providing strong evidence of Dmrt5's participation in that collaboration. The complexity of the interaction between ventral midbrain regulators may go some way to explaining the incomplete blockade of mDA production in loss-of-function mouse models. With the exception of Shh, which is required early for any ventral cell fate, mutations of these genes singly result only in delay or partial depletion of mDA neurons, perhaps indicating a robust system of compensation for a lack in any individual part of these densely interacting cascades (10, 11, 26).

A major challenge faced by stem cell research is to assign the correct patterning code to progenitors of interest. In normal development, ventral neural progenitors establish their regional specification by responding to graded Shh signaling (13). However, Shh's ability to induce the mDA phenotype is temporally and context-dependent (6, 23), and other ventral cell types, such as hindbrain serotonergic neurons are induced under a similar scheme (16, 17). This complexity could explain the "misspecification" of ventral progenitors in ESC cultures shown by coexpression of Foxa2 with Nkx2.2, Lhx1, or Isl1. The immunostaining and global gene-expression analysis shown here demonstrates that Dmrt5 guards the medial domain identity by restricting ventral progenitors to an accurate medial mDA fate. Given that acquisition of midbrain regional identity is critical for successful functional integration in cell therapy (29), our finding that Dmrt5 enforces a midbrain fate would have important implications in directed stem cell differentiation.

In addition to confirming a regulatory role for Dmrt5 in cell-fate specification, our global gene-expression analysis provides a valuable platform of knowledge from which to gain further insights on factors and molecular cascades that participate in Dmrt5-mediated cell-fate decisions.

Materials and Methods

ESC Lines. The generation of ESC lines that conditionally overexpress Dmrt5 or Dmrt5 shRNA are described in *SI Materials and Methods*. Two independent Dmrt5-overexpressing and shRNA knockdown lines were used in this study and gave similar results. PCR primer list is provided in Table S1.

Neural Differentiation, Immunocytochemistry, and In Situ Hybridization. Routine ESC culture and monolayer neural differentiation was performed as described in refs. 22 and 23. The schemes for conditional Dmrt5 overexpression and knockdown, detailed antibody staining, and in situ hybridization are described in *SI Materials and Methods*.

Microarray Analysis. Detailed sample preparation and analysis of the microarray data are provided in *SI Materials and Methods*. Three biological replicates of the paired samples were generated. Labeled cRNAs were generated from 10 ng of total RNA by the T7-based one-cycle protocol and hybridized to Affymetrix MOE430.2 GeneChips per the manufacturer's instructions. Microarray data in CEL format were analyzed with the GeneSpring GX11 program.

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1. Ang SL (2006) Transcriptional control of midbrain dopaminergic neuron development. *Development* 133:3499–3506.
2. Zhao S, et al. (2004) Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons. *Eur J Neurosci* 19:1133–1140.
3. Joksimovic M, et al. (2009) Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nat Neurosci* 12:125–131.
4. Chung S, et al. (2009) Wnt1-lmx1a forms a novel autoregulatory loop and controls midbrain dopaminergic differentiation synergistically with the SHH-FoxA2 pathway. *Cell Stem Cell* 5:646–658.
5. Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* 93:755–766.
6. Andersson E, et al. (2006) Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* 124:393–405.
7. Heine P, Dohle E, Bumsted-O'Brien K, Engelkamp D, Schulte D (2008) Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development. *Development* 135:805–811.
8. Mariani M, et al. (1998) Mab21, the mouse homolog of a *C. elegans* cell-fate specification gene, participates in cerebellar, midbrain and eye development. *Mech Dev* 79:131–135.
9. Nakatani T, Minaki Y, Kumai M, Ono Y (2007) Helt determines GABAergic over glutamatergic neuronal fate by repressing Ngn genes in the developing mesencephalon. *Development* 134:2783–2793.
10. Puelles E, et al. (2004) Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* 131:2037–2048.
11. Nakatani T, Kumai M, Mizuhara E, Minaki Y, Ono Y (2010) Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon. *Dev Biol* 339:101–113.
12. Lin W, et al. (2009) Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development. *Dev Biol* 333:386–396.
13. Briscoe J, et al. (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* 398:622–627.
14. Hong CS, Park BY, Saint-Jeannet JP (2007) The function of Dmrt genes in vertebrate development: It is not just about sex. *Dev Biol* 310:1–9.
15. Huang X, Hong CS, O'Donnell M, Saint-Jeannet JP (2005) The doublesex-related gene, *XDmrt4*, is required for neurogenesis in the olfactory system. *Proc Natl Acad Sci USA* 102:11349–11354.
16. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* 18:675–679.
17. Barberi T, et al. (2003) Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol* 21:1200–1207.
18. Sonntag KC, Simantov R, Kim KS, Isacson O (2004) Temporally induced Nurr1 can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation. *Eur J Neurosci* 19:1141–1152.
19. Maxwell SL, Ho HY, Kuehner E, Zhao S, Li M (2005) Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Dev Biol* 282:467–479.
20. Konstantoulas CJ, Parmar M, Li M (2010) FoxP1 promotes midbrain identity in embryonic stem cell-derived dopamine neurons by regulating Pitx3. *J Neurochem* 113:836–847.
21. Kim JH, et al. (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50–56.
22. Ying QL, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 21:183–186.
23. Parmar M, Li M (2007) Early specification of dopaminergic phenotype during ES cell differentiation. *BMC Dev Biol* 7:86.
24. Xiang M, Gan L, Zhou L, Klein WH, Nathans J (1996) Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. *Proc Natl Acad Sci USA* 93:11950–11955.
25. Ventura A, et al. (2004) Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci USA* 101:10380–10385.
26. Blaess S, Corrales JD, Joyner AL (2006) Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. *Development* 133:1799–1809.
27. Prakash N, et al. (2006) A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* 133:89–98.
28. Jacob J, et al. (2007) Transcriptional repression coordinates the temporal switch from motor to serotonergic neurogenesis. *Nat Neurosci* 10:1433–1439.
29. Hudson JL, Bickford P, Johansson M, Hoffer BJ, Strömberg I (1994) Target and neurotransmitter specificity of fetal central nervous system transplants: Importance for functional reinnervation. *J Neurosci* 14:283–290.