Development 137, 1897-1906 (2010) doi:10.1242/dev.042572

The basic helix-loop-helix transcription factor Nato3 controls neurogenic activity in mesencephalic floor plate cells

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SUMMARY

Floor plate (FP) cells, the ventral midline cells of the developing neural tube, have long been thought to be non-neurogenic organizer cells that control neuronal patterning and axonal guidance. Recent studies have revealed that mesencephalic FP (mesFP) cells have neurogenic activity and generate dopaminergic neurons. However, the mechanisms underlying the control of neurogenic potential in FP cells are not yet fully understood. Here we identified the bHLH factor Nato3 as an FP-specific transcription factor. In *Nato3*-null mutant mice, FP cells in the spinal cord were correctly specified, but could not properly mature. By contrast, in the developing mesencephalon, loss of *Nato3* did not affect FP differentiation, but led to loss of neurogenic activity in the medial subpopulation of mesFP cells by suppressing proneural gene expression and inducing cell cycle arrest. As a consequence, the number of midbrain dopaminergic neurons generated was decreased in mutants. We also found that Hes1, which is known to be required for non-dividing organizer cell development in the neural tube, was aberrantly upregulated in the mesFP cells of *Nato3* mutants. Consistently, forced expression of *Nato3* repressed Hes1 expression and consequently induced premature neurogenesis. Finally, we showed that forced expression of *Hes1* in mesFP cells induced cell cycle arrest and downregulation of proneural factors. Taken together, these results suggest that *Nato3* confers neurogenic potential on mesFP cells by suppressing classical non-neurogenic FP cell differentiation, at least in part, through repressing Hes1.

KEY WORDS: Nato3, Floor plate cell, Neurogenic activity, Dopaminergic neuron, Mesencephalon, Anteroposterior identity, Mouse

INTRODUCTION

Floor plate (FP) cells are morphologically specialized cell populations that develop at the ventral midline of the neural tube (Placzek and Briscoe, 2005; Strahle et al., 2004). FP cells organize ventral cell fate patterning and the projection of commissural axons by secreting diffusible factors such as Shh and netrin 1 and contacting axons via cell adhesion molecules. Shh secreted from underlying axial mesodermal tissues is known to specify FP cells by activating Gli2 (Chiang et al., 1996; Matise et al., 1998; Roelink et al., 1995). The FP cell-selective transcription factor Foxa2 is thought to be involved in this specification process by acting as a downstream effector of Shh signaling because ectopic expression of *Foxa2* can induce FP cell differentiation (Sasaki and Hogan, 1994). However, expression of Foxa2 is not specific to FP cells; it is also expressed in the neural progenitors neighboring FP cells throughout the neural tube (Ono et al., 2007), suggesting the possible existence of another transcription factor that strictly determines FP cell identity.

FP cells have long been thought to be non-proliferative cells that never give rise to neurons by themselves (Jessell, 2000; Placzek and Briscoe, 2005). Consistent with this non-neurogenic property of FP cells, persistent expression of Hes1, which suppresses proneural gene expression, is required for the establishment of FP cell fate (Baek et al., 2006). However, recent cell-sorting and lineage-tracing studies revealed that FP cells in the developing mesencephalon have neurogenic potential and indeed generate mesencephalic dopaminergic (mesDA) neurons (Bonilla et al., 2008; Kittappa et al., 2007; Ono et al., 2007). Thus, FP cells that develop at different anteroposterior (AP) locations have different characteristics: non-neurogenic classical FP cells in the caudal neural tube and neurogenic FP cells in the mesencephalon (hereafter referred to as cFP and mesFP cells, respectively).

Recently it was proposed that Wnt signaling-mediated downregulation of Shh, which suppresses neurogenic potential, confers neurogenic activity on FP cells (Joksimovic et al., 2009a). In addition to this activity, Wnt1 has been shown to control expression of *Otx2*, a master determinant of the mesencephalic identity of FP cells that confers proliferative and neurogenic potential, in FP cells (Brodski et al., 2003; Omodei et al., 2008; Ono et al., 2007; Prakash et al., 2006; Puelles et al., 2004; Vernay et al., 2005). In FP cells, Otx2 induces Lmx1a expression, which in turn induces Msx1, and both of these transcription factors are required for proper proneural gene expression and subsequent neurogenesis in the mesFP cells (Andersson et al., 2006b; Omodei et al., 2008; Ono et al., 2007). Thus, this transcription factor cascade appears to determine the neurogenic activity of the mesFP cells. However, *Otx2*, but not *Lmx1a*, is sufficient for conferring neurogenic activity on cFP cells (Ono et al., 2007), suggesting that *Otx2* functions in other pathway(s) as well. In addition, an FP cell fate determinant, Foxa2, which is not selectively expressed in FP cells of the mesencephalon, has been shown to be involved in the regulation of neurogenesis in mesFP cells (Ferri et al., 2007). Therefore, the complex extrinsic and intrinsic signaling pathways determining neurogenic mesFP or cFP cell identity have not yet been fully unmasked.

In the present study, we identified a novel regulator of FP development, *Nato3*. In the caudal neural tube, *Nato3* is required for proper development of FP cells. By contrast, in the mesencephalon, *Nato3* activity is largely dispensable for FP

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Accepted 1 April 2010

differentiation but is required for cell cycle progression and induction of proneural genes in medial FP cells, at least in part, by repressing Hes1. In addition, loss of *Nato3* leads to cFP cell-like differentiation of mesencephalic ventral midline cells. Thus, our observations suggest that *Nato3* is involved in the determination of the AP identity of FP cells and that suppression of cFP cell differentiation pathway by *Nato3* is essential for the acquisition of neurogenic potential by mesFP cells.

MATERIALS AND METHODS

Mice

A *Nato3* targeting vector was assembled using pGFP-neo-DT-A, which contains the GFP cDNA and neomycin gene cassettes in a Bluescript SK+ (Stratagene) backbone. Genomic sequences encompassing the mouse *Nato3* gene were isolated from a C57Bl/6 genomic phage library. A 2 kb 5′ arm-containing genomic fragment just upstream of the initiation codon of *Nato3* and a 7.5 kb 3′ arm-containing fragment were separately cloned into pGFP-neo-DT-A to generate the *Nato3* targeting vector. *Nato3*-null mice were generated by homologous recombination in the C57Bl/6 embryonic stem cell line according to standard procedures, and germline transmission of the mutation was confirmed by Southern blotting and PCR. *Nato3*$^{-/-}$ mice were generated by crossing heterozygous mutant mice on a C57Bl/6 background and were genotyped by PCR.

Dreher^{J} (*Lmx1a* – Mouse Genome Informatics) mice (Millonig et al., 2000) were obtained from the Jackson Laboratory and maintained as previously described (Ono et al., 2007).

Transgenic constructs were obtained by ligating each cDNA amplified by PCR into the pNE vector (Nakatani et al., 2007). The primer sequences used for amplification of the cDNA fragments are available upon request. Linearized pNE constructs were injected into fertilized eggs from C57Bl/6 mice and founder embryos were collected at the indicated stages. The embryos were genotyped by PCR and tested for transgene expression by immunostaining. We chose transgenic embryos expressing transgenes at similar levels for further analyses and observed essentially the same phenotypes in all chosen embryos. The numbers of transgenic embryos analyzed were as follows: NE-Nato3, *n*=7; NE-Hes1, *n*=7; NE-Foxa2, *n*=3; NE-Shh, *n*=3.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described previously (Nakatani et al., 2004). Rat anti-Nato3 mAb was raised against GST-Nato3 [amino acids (aa) 1-95]. Armenian hamster anti-Dbx1 and anti-Lmx1b mAbs were raised against GST-Dbx1 (aa 247-335) and GST-Lmx1b (aa 271-306). A polyclonal rabbit anti-Olig2 antibody was raised against GST-Olig2 (aa 1-47) and affinity-purified. Other primary antibodies used in this study included the following: anti-Corin, anti-Lmx1a, anti-Pitx3, anti-Nkx6.1 and anti-Nurr1 (Ono et al., 2007); anti-Shh, anti-En1, anti-Pax6, anti-Msx1/2 and anti-Nkx2.2 (Developmental Studies Hybridoma Bank); anti-HuC/D (Molecular Probes); anti-Lhx1, anti-Sox2, anti-Ngn1, anti-Ngn2, anti-Hes1 and anti-Foxa2 (Santa Cruz Biotechnology); anti-TH and anti-Brn3a (Chemicon); anti-Mash1 and anti-p27Kip1 (BD Pharmingen); anti-Ki67 (Novocastra); anti-Shh, anti-Otx2 and anti-BrdU (Abcam); and anti-caspase 3 (Cell Signaling).

In situ hybridization was performed as described previously (Nakatani et al., 2004). The primer sequences used for amplification of probe cDNAs (*Nato3*, *Tem7r*, *Sim1*, *Metrnl*, *BMP1*, *SCF*, annexin A2 and vitronectin) are available upon request.

RESULTS

Nato3 is selectively expressed in FP cells

To identify genes that regulate FP and mesDA development we searched for genes selectively expressed in FP cells by comparing gene expression levels in the ventral midline region and in the basal plate region of the developing mesencephalon by subtractive PCR (Ono et al., 2007). One of the cDNA fragments obtained encoded the basic helix-loop-helix (bHLH) transcription factor Nato3 (also known as N-Twist or Ferd3l – Mouse Genome Informatics) (Segev et al., 2001). We first examined the expression pattern of *Nato3* in E11.5 embryos by in situ hybridization. In the spinal cord, *Nato3* was specifically expressed in the ventral midline (Fig. 1A), as previously reported (Verzi et al., 2002). Ventral midline-specific expression was also observed in the hindbrain, mesencephalon and caudal diencephalon (Fig. 1A). *Nato3* expression was not detected in more-rostral brain regions at this stage (data not shown). Essentially the same pattern of expression was observed by immunohistochemistry using an anti-Nato3 monoclonal antibody (Fig. 1A), indicating the specificity of both staining methods.

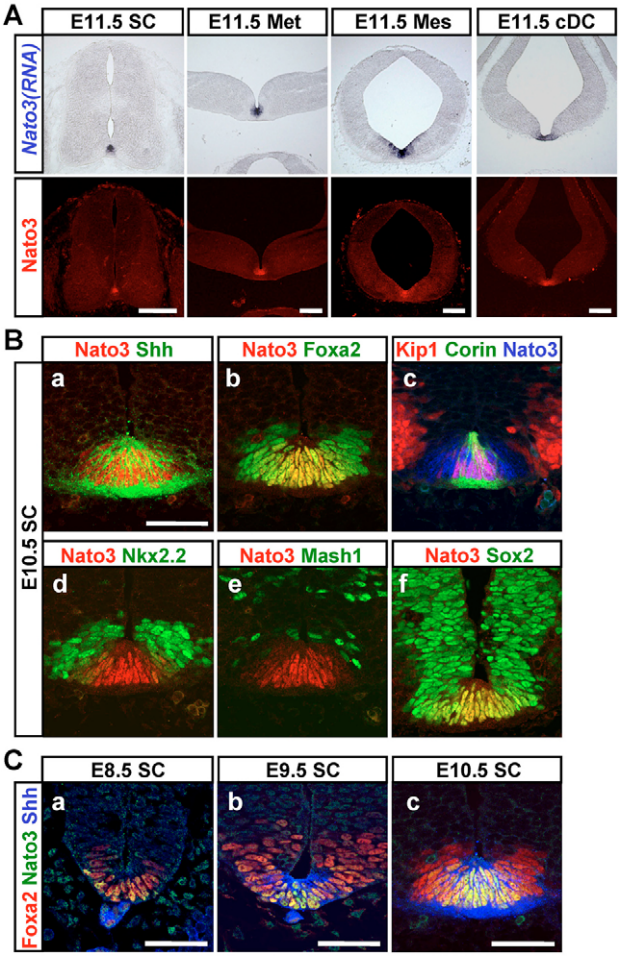

Fig. 1. Nato3 is selectively expressed in FP cells. (**A**) Expression of Nato3 in the developing neural tube. The images show serial sections of the E11.5 mouse spinal cord (SC), metencephalon (Met), mesencephalon (Mes) and caudal diencephalon (cDC) regions hybridized with a *Nato3* antisense riboprobe (upper panels) or stained with an anti-Nato3 antibody (lower panels). (**B**) Floor plate (FP)-specific expression of Nato3 in the developing spinal cord. Nato3 is co-expressed with Shh (a) and Foxa2 (b) but not with proneural factor Mash1 (e). The medial subpopulation of Nato3^{+} cells (medial FP) selectively expresses p27Kip1 and Corin (c), whereas the lateral subpopulation (lateral FP) expresses a low level of Nkx2.2 (d). All Nato3^{+} cells express Sox2 (f). (**C**) Expression of Nato3 in the spinal cord FP in the early developmental stages. At E8.5, a low level of Nato3 expression can be detected at the ventral midline (a). From E9.5 onwards, Nato3 is specifically expressed in the FP cells at a high level (b,c). Scale bars: 200 μm in A; 50 μm in B,C.

To determine the identity of cells expressing Nato3, expression of several regional markers in Nato3^{+} cells was examined. In the spinal cord, Nato3 was co-expressed with FP markers, such as Shh and Foxa2, at E10.5 (Fig. 1Ba,b). Importantly, Nato3 expression was medially restricted within cells positive for Foxa2. A high level of Nato3 expression was detected in definitive FP cells, as judged by the expression of p27Kip1 (Cdkn1b – Mouse Genome Informatics) and Corin (Fig. 1Bc) with prolonged nuclear shapes (Fig. 1Bb). A lower expression level was detected in the Nkx2.2^{low} Foxa2^{high} lateral FP cells that were negative for proneural factors (Fig. 1Bd,e). All Nato3^{+} FP cells expressed Sox2 (Fig. 1Bf). Similar FP-specific expression of Nato3 was observed in the hindbrain (data not shown).

We next examined Nato3 expression in the early stages of development. At E8.5, when the ventral midline cells acquire midline characteristics such as expression of Foxa2 and Shh but are still proliferative, as indicated by expression of Ki67 (Mki67 – Mouse Genome Informatics; Fig. 5B), Nato3 expression was observed in the medial subpopulation of FP cells (Fig. 1Ca). Thus, the onset of Nato3 expression in the FP cells precedes FP cell differentiation. The expression level was increased at E9.5 and, thereafter, Nato3 expression persisted at least until E14.5 (Fig. 1Cb,c; data not shown).

FP cells develop at the ventral midline of the developing brain from the caudal diencephalon to the spinal cord (Placzek and Briscoe, 2005). However, the FP cells in the mesencephalon and those in the caudal neural tube have different characteristics, such as differing gene expression profiles and neurogenic activities (Ono et al., 2007; Placzek and Briscoe, 2005). Therefore, we next examined the precise expression pattern of Nato3 in the developing mesencephalon. In the mesencephalon, FP cells can be identified by the expression of Shh in the early stage, but as development proceeds, Shh expression expands into lateral red nucleus (RN) domains (Andersson et al., 2006b; Joksimovic et al., 2009b). Instead, Corin and Lmx1a specifically mark FP cells at later stages (Ono et al., 2007). At E11.5, a high level of Nato3 expression was observed in the medial part of the FP cell domain, which was positive for Lmx1a and Corin (Fig. 2Aa,b). A lower expression level was observed in the lateral part of the FP domain and the ventral part of the RN domain. All Nato3^{+} cells were localized to the ventricular zone (VZ) and expressed the proliferative neural progenitor markers Sox2 and Ki67, but not p27Kip1, at a high level, in contrast to the observations in the caudal neural tube midline (Fig. 2Ac-e). In addition, a subset of Nato3^{+} cells expressed the proneural factors Ngn2 (Neurog2 – Mouse Genome Informatics) and Mash1 (Ascl1 – Mouse Genome Informatics; Fig. 2Af,g). It should be noted that progenitor cells in the VZ expressing high levels of proneural genes showed relatively lower levels of Nato3 expression (see Fig. S1 in the supplementary material). HuC/D (Elavl3/4 – Mouse Genome Informatics)-positive and p27Kip1^{high} neurons emerging from these progenitors showed no Nato3 expression (Fig. 2Ah). These observations indicate that Nato3 is specifically expressed by mesDA progenitors in the E11.5 mesencephalon and suggest that Nato3 becomes downregulated early in the neuronal differentiation process in nascent postmitotic mesDA precursors.

At E8.5, Nato3 expression was readily detectable in a small cluster of cells at the ventral midline of the mesencephalon (Fig. 2Ba). At this stage, a mesDA fate determinant, Lmx1a, was not expressed in this region. One day later, Nato3 expression expanded laterally within the FP region defined by Shh and Lmx1a expression (Fig. 2Bb). FP-selective expression of Nato3 continued until E10.5, although at this later stage, the expression level declined in the lateral part of the FP region (Fig. 2Bc). After E11.5, graded Nato3 expression expanded into the RN domain and persisted until E18.5 (Fig. 2Aa; data not shown).

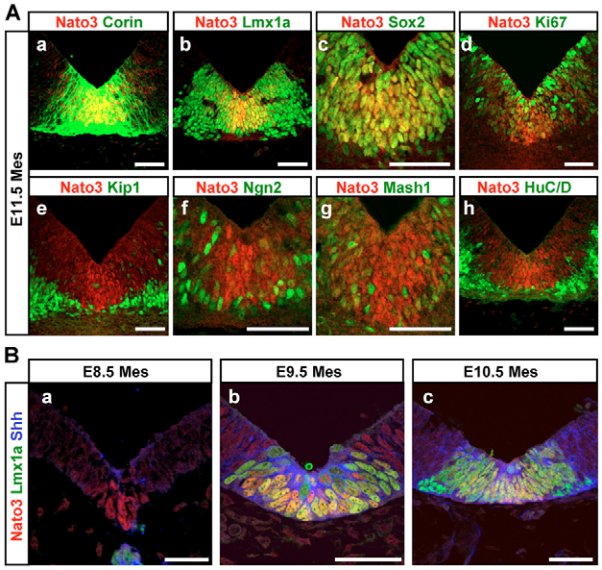

Fig. 2. Nato3 is selectively expressed in FP/mesDA progenitors in the developing mesencephalon. (**A**) Nato3 is selectively expressed in mesFP cells positive for Corin (a) and Lmx1a (b) at E11.5. The medial subpopulation of mesFP expressed Nato3 at a high level. All Nato3^{+} cells expressed Sox2 (c) and Ki67 (d) but not p27Kip1 (e) or HuC/D (h). A subpopulation of Nato3^{+} cells expressed the proneural factors Ngn2 (f) and Mash1 (g). (**B**) Expression of Nato3 in mesFP cells in the early developmental stages. A small cluster of cells at the ventral midline expressed Nato3 and Shh at E8.5, before the onset of Lmx1a expression (a). At E9.5, floor plate cells defined by Shh and Lmx1a expression specifically expressed Nato3 at a high level (b). One day later, Nato3 expression in the lateral part of the FP region began to decline (c). Scale bars: 50 μm.

Taken together, these results showed that Nato3 is highly selectively expressed by the FP cells in the developing neural tube. In addition, the early onset of Nato3 expression in FP cells suggests its involvement in FP cell specification and/or differentiation.

Nato3 is required for correct differentiation of FP cells in the caudal neural tube

Previous in vitro studies have suggested that Nato3 inhibits the transcriptional activity of Mash1 by sequestrating E proteins (Verzi et al., 2002). However, to the best of our knowledge, the in vivo role of Nato3 has yet to be analyzed. To examine the role of *Nato3* in FP cell specification and/or differentiation, we generated *Nato3*-null mutant mice by targeted disruption (see Fig. S2 in the supplementary material). Homozygous mutant mice were morphologically normal and could survive until adulthood (data not shown). We first examined the effect of the *Nato3* mutation on FP cell differentiation in the spinal cord. At E10.5, p27Kip1^{+} cell-cycle-arrested FP cells were generated in the ventral midlines of wild-type embryos (Fig. 3Aa). In *Nato3*-null embryos, p27Kip1^{+} midline cells were generated normally (Fig. 3Aa′). Expression of Foxa2 and Shh was not affected, and midline cells were devoid of proneural gene expression (Fig. 3Aa-b′; data not shown). In addition, the ventral patterning of

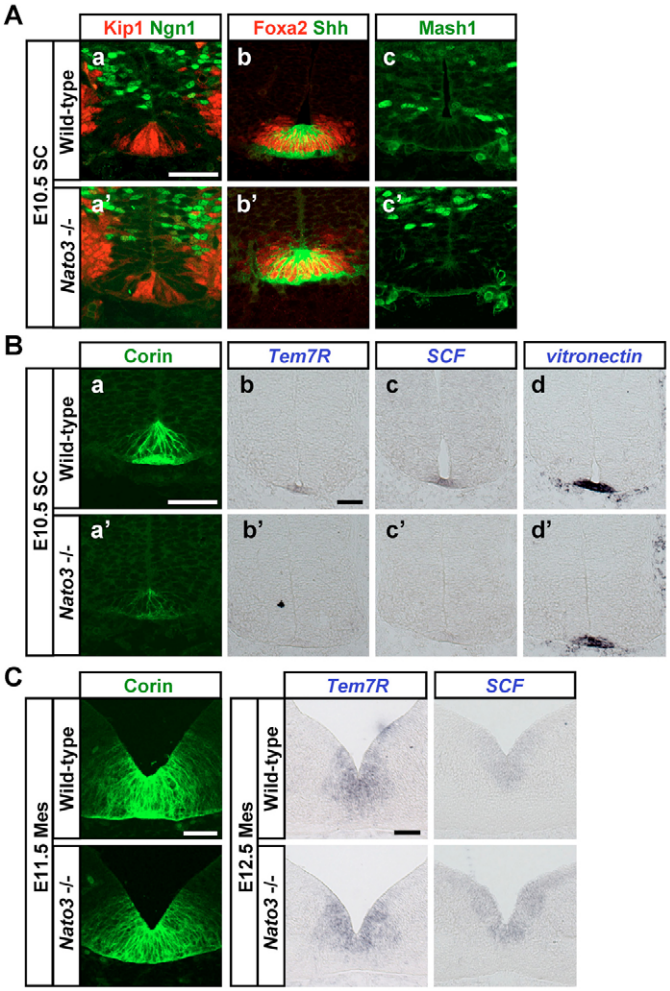

Fig. 3. *Nato3* is required for correct differentiation of FP cells in the spinal cord but not in the mesencephalon. (**A**) FP cells are normally specified in the absence of *Nato3*. p27Kip1$^+$ cell-cycle-arrested FP cells (a,a′) positive for Foxa2 and Shh (b,b′) but negative for Ngn1 (a,a′) and Mash1 (c,c′) are normally generated in *Nato3* mutant spinal cords. (**B**) Medial FP-selective genes, including Corin (a,a′), *Tem7R* (b,b′) and *SCF* (c,c′), are downregulated in the spinal cord FP cells of *Nato3* mutants. By contrast, the lateral FP gene vitronectin is only slightly affected (d,d′). (**C**) FP cell differentiation is not affected by loss of *Nato3* in the developing mesencephalon. Scale bars: 50 μm.

the spinal cord in mutants was also normal (see Fig. S3 in the supplementary material), indicating that FP cells were specified normally. To examine whether FP cells could differentiate normally in the absence of *Nato3*, we analyzed the expression of other marker genes selectively expressed in FP cells, including Corin, *Tem7r* (*Plxdc2* – Mouse Genome Informatics), *SCF* (*Kitl* – Mouse Genome Informatics), *BMP1*, annexin A2 (*Anxa2* – Mouse Genome Informatics), *Metrnl* and vitronectin (*Vtn* – Mouse Genome Informatics) (Gore et al., 2008; Miller et al., 2007; Sasaki and Hogan, 1994; Seiffert et al., 1995). Among these markers, the expression levels of medial FP-selective markers such as Corin, *Tem7r* and *SCF* were significantly lower in *Nato3* mutants than controls (Fig. 3Ba-c′). By contrast, FP markers expressed both in lateral and medial FP cells, including *BMP1*, annexin A2, *Metrnl* and vitronectin, were only slightly affected (Fig. 3Bd,d′; data not shown). The decrease in marker expression levels continued at least until E12.5, although the difference in expression level compared with wild-type controls became smaller (data not shown), suggesting that correct FP cell maturation could not occur in the absence of *Nato3*, and that this was not caused by simple delay of differentiation. At present, the significance of these defects in FP function caused by *Nato3* mutation, such as axonal guidance, is unclear because commissural axons appeared to cross the ventral midline normally; however, we have not examined precisely the pattern of axonal projection (data not shown). Importantly, FP differentiation in the mesencephalon was not affected by loss of *Nato3* given that FP marker expression was mostly normal in mutants (Fig. 3C; data not shown). Thus, *Nato3* is required for correct FP maturation only in the caudal neural tube.

Nato3 controls mesDA neuron generation

The early onset of Nato3 expression in the mesencephalon suggests a possible involvement of *Nato3* in regional patterning or mesDA progenitor specification. To test this possibility, we analyzed regional marker expression in *Nato3* mutants. At E11.5, the mesDA progenitor markers Lmx1a and Msx1/2 were normally expressed in mutants and expression of neighboring RN progenitor markers Nkx6.1 and *Sim1* was also unaffected by loss of *Nato3* (see Fig. S4A in the supplementary material), suggesting that *Nato3* is not involved in ventral patterning in the mesencephalon and mesDA progenitor specification. We next asked whether *Nato3* activity is required for mesDA neuron specification. At E12.5, postmitotic mesDA neuron markers, including Lmx1a/b, En1, Pitx3, Nurr1 (Nr4a2 – Mouse Genome Informatics) and TH, were normally expressed in the postmitotic neurons emerging from the midline of the mutant mesencephalon, and these neurons did not ectopically express the neighboring RN markers Lhx1 and Brn3a (Pou4f1 – Mouse Genome Informatics; see Fig. S4Ba-d′ in the supplementary material). In addition, virtually all neurons generated from the FP region were specified to become Nurr1$^+$ mesDA neurons in the mutants, as in wild-type embryos (see Fig. S4Be,e′ in the supplementary material). Therefore, even in the absence of *Nato3*, m7 neurons were correctly specified as mesDA neurons. However, we observed a significant reduction in the number of mesDA neurons in the *Nato3* mutant embryos compared with wild-type controls.

We analyzed the number and localization of mesDA neurons in sections throughout the entire mesencephalon at E12.5. We found that the number of mesDA neurons located near the midline within the mesDA domain was significantly reduced in both the anterior and posterior mesencephalon (Fig. 4A; see also Fig. S5 in the supplementary material). Laterally localized mesDA neurons were relatively less affected. We counted the number of Pitx3$^+$ TH$^+$ mesDA neurons at E14.5, when most mesDA neurons had differentiated into postmitotic neurons. Consistent with the observation at E12.5, the number of mesDA neurons was decreased to 67% in *Nato3* mutant embryos (Fig. 4B,D; see also Fig. S5 in the supplementary material). By contrast, the number of RN neurons was not significantly affected (heterozygous control, 920.3±31.8 cells/section; mutants, 983.0±15.9 cells/section; see Fig. S6 in the supplementary material).

To examine whether this phenotype is caused by a delay in mesDA neurogenesis, we analyzed *Nato3* mutant mice at E18.5 and in adulthood (see Fig. S7 in the supplementary material; data not shown). A consistent level of reduction in the mesDA neuron number was observed at both stages. Thus, continuous generation

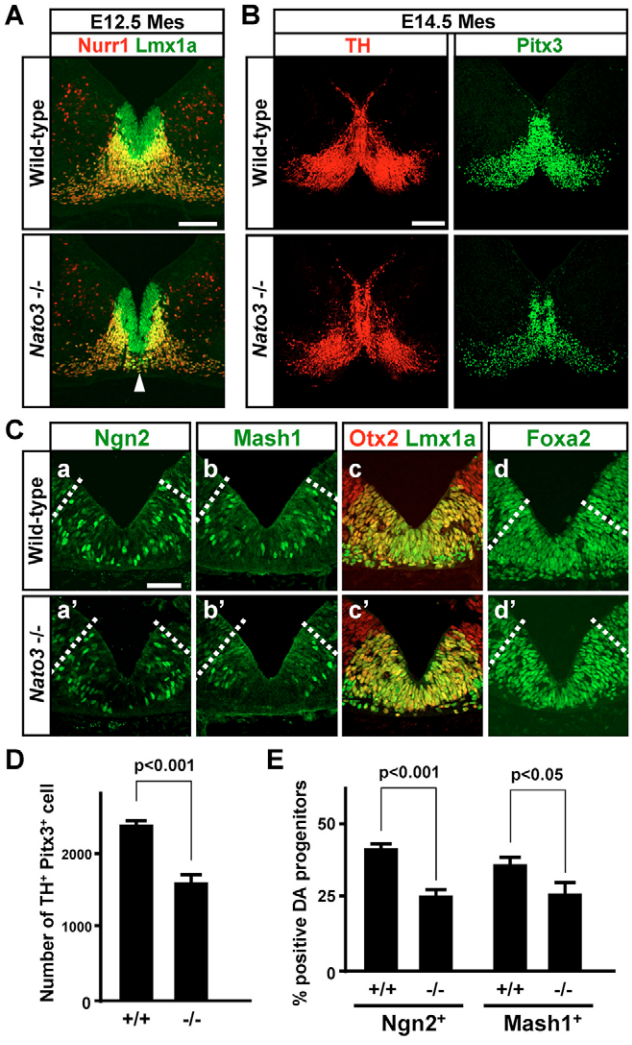

Fig. 4. *Nato3* regulates mesDA neurogenesis in mesFP cells. (**A**) Medially located mesDA neurons are reduced in the *Nato3* mutant at E12.5 (arrowhead). (**B**,**D**) The mesDA number is reduced in *Nato3* mutant embryos compared with wild-type embryos at E14.5. Quantification of TH$^+$ Pitx3$^+$ neurons in the ventral mesencephalons of wild-type and *Nato3* mutant embryos at E14.5 is shown in D. (**C**,**E**) Expression of proneural factors Ngn2 (a,a′) and Mash1 (b,b′) is reduced in mesFP cells, especially in the medial subpopulation of the *nato3* mutant at E11.5. Expression of known neurogenesis regulators in mesFP cells, namely, Otx2, Lmx1a (c,c′) and Foxa2 (d,d′), is unaffected by the loss of *Nato3*. Dotted lines indicate the m6/m7 boundaries defined by Lmx1a expression (data not shown). Quantification of Ngn2$^+$ and Mash1$^+$ DA progenitors in wild-type and *Nato3* mutant embryos at E11.5 is shown in E. Scale bars: 100 μm in A; 200 μm in B; 50 μm in C.

of mesDA neurons at the later stage cannot compensate for the defect, ruling out the possibility that mesDA neurogenesis is delayed by loss of *Nato3*. It should be noted that similar levels of reduction in mesDA neuron number were observed in the SNc (substantia nigra compacta) and VTA (ventral tegmental area) of adult mutant mice, and that the A9 (Girk2$^+$)/A10 (calbindin$^+$) subtype ratio was not significantly changed in the *Nato3* mutants despite the clear difference in the severity of neurogenesis defects induced by *Nato3* mutation between lateral and medial mesDA progenitor subpopulations (data not shown).

Nato3 is required for proliferation and neurogenic potential in mesFP cells

The above observations indicated that the defect in mesDA differentiation is not a cause of the phenotype in the *Nato3* mutant. As in *Pitx3* and *En1/2* KO mice (Alberi et al., 2004; Smidt et al., 2004; van den Munckhof et al., 2003), cell death could be responsible for the decrease in the number of mesDA neurons. However, active caspase 3 staining revealed that apoptosis was not accelerated in *Nato3* mutants at E12.5 and E14.5 (see Fig. S8 in the supplementary material). Therefore, the defect in neurogenesis appears to be a cause of the phenotype.

To examine this possibility, we first analyzed the expression of the proneural factors Ngn2 and Mash1, which are required for mesDA generation (Andersson et al., 2006a; Kele et al., 2006), at E11.5. A significant reduction in proneural factor expression was observed in the medial subpopulation of mesDA progenitors (Fig. 4Ca-b′), which highly paralleled the pattern of reduction in mesDA neuron number. The percentages of mesDA progenitors expressing Ngn2 and Mash1 in the *Nato3* mutants were reduced from 39.8% ± 1.5% and 33.9% ± 3.2% to 25.4% ± 2.4% and 25.8% ± 3.4%, respectively (Fig. 4E). Taken together, these results suggest that Nato3 is required for efficient neurogenesis in mesFP cells, and that reduced neurogenic activity is a cause of the mesDA neuron reduction in *Nato3* mutant mice.

Because mesDA neurons are generated from FP cells, which are non-proliferative in the caudal neural tube, and the mesFP factor Otx2 confers proliferative potential on FP cells that consequently induce mesDA neurogenesis (Omodei et al., 2008; Ono et al., 2007), we next examined the proliferation properties of mesFP cells in the *Nato3* mutants. At E12.5, almost all mesDA progenitors in wild-type embryos were positive for the proliferation marker Ki67 and negative for p27Kip1 (Fig. 5Aa,b). By contrast, in the *Nato3* mutants, the medial subpopulation of the FP cells ectopically expressed p27Kip1, and Ki67 was consistently downregulated in these cells (Fig. 5Aa′,b′), indicating that these cells have exited the cell cycle. These p27Kip1$^+$ cells in the VZ maintained Sox2 expression, like caudal FP cells (Fig. 5Ac′), ruling out the possibility of ectopic localization of neurons in the mutants. Furthermore, the expression levels of *Shh*, vitronectin and annexin A2, all of which are selectively expressed in caudal FP cells at a high level, were significantly increased in the medial mesFP cells of *Nato3* mutants, as in caudal FP cells of wild-type embryos at E12.5 (Fig. 5Ad-e′; data not shown). Taken together, these results suggest that ventral midline cells in the mesencephalon acquire a cFP cell-like identity that suppresses proliferation and neurogenic potential in *Nato3* mutants.

The phenotype in mesFP cells caused by loss of *Nato3* activity was highly similar to that reported for mesFP cells in *Otx2* cKO mice (Omodei et al., 2008), suggesting the possible involvement of *Nato3* in the maintenance of Otx2 expression in mesFP cells. However, Otx2 was expressed normally in *Nato3* mutant embryos (Fig. 4Cc,c′). Similarly, expression of other transcription factors controlling mesDA neurogenesis, namely Lmx1a, Msx1/2 and Foxa2, was not affected by loss of *Nato3* (Fig. 4Cc-d′; data not shown). Therefore, neurogenic differentiation of mesFP cells is controlled by *Nato3* by some mechanism other than regulating the expression of previously identified transcription factors required for neurogenic activity in mesFP cells.

It has been proposed that non-neurogenic FP cells exist in the mesencephalon in the early stage of development (E9.75) and, later, that mesFP cells convert to neural progenitors by acquiring proneural gene expression (Andersson et al., 2006b). However,

a recent study showed that mesFP cells incorporated BrdU at E9.75 (Joksimovic et al., 2009a); consistently, we could not observe any p27Kip1$^+$ FP cells in the mesencephalon at any of the stages examined (Fig. 5Ba-d). In *Nato3* mutants, as in wild-type controls, p27Kip1$^+$ FP cells were not detected until E10.5 (Fig. 5Ba′,b′). Thereafter, however, midline cells started to lose Ki67 expression and express p27Kip1 in the mutants (Fig. 5Bc′,d′). This FP differentiation regarding cell cycle exit of the midline cells in the mutant mesencephalon is similar to the case in the midline of the spinal cords of wild-type embryos (Fig. 5Ba′-d′,e-h). Therefore, Nato3 appears to be required for cell cycle progression in medial mesFP cells. To further confirm this idea, we performed BrdU pulse-chase labeling experiments in which BrdU was injected at E10.75 and labeled cells were analyzed at E12.5 (see Fig. S9 in the supplementary material). In wild-type embryos, a similar number of BrdU$^+$ Ki67$^+$ Sox2$^+$ VZ cells, which represent cells that had incorporated BrdU at E10.75 and re-entered the cell cycle, were observed in the lateral and medial mesFP regions. One- to two-fold more BrdU$^+$ Ki67$^-$ Sox2$^-$ cells existed in the mantle layer and these probably represent postmitotic neurons generated from the FP, suggesting that, during the chase period (E10.75-E12.5), a few rounds of asymmetric divisions occurred in BrdU-labeled mesFP cells to generate neurons. In the lateral part of the mutant mesFP, the labeling pattern was similar to that in wild-type controls. By contrast, in the medial part of the mutant mesFP, the number of BrdU$^+$ Ki67$^+$ Sox2$^+$ cells was significantly reduced (wild-type, 56.9±8.9 cells/section; mutants, 27.8±8.8 cells/section, P=0.015) and the number of cFP-like BrdU$^+$ Ki67$^-$ Sox2$^+$ cells was increased (wild-type, 13.3±4.6 cells/section; mutants, 24.4±8.4 cells/section). The number of total Sox2$^+$ BrdU$^+$ cells, which reside within the VZ, was not significantly changed (wild-type, 70.2±6.9 cells/section; mutants, 52.2±10.3 cells/section). These results demonstrate that, in the absence of *Nato3*, a significant portion of medial mesFP cells, which had proliferated at E10.75, exited the cell cycle but were still retained within the VZ to differentiate into cFP-like cells by E12.5. Consistently, the number of BrdU$^+$ Ki67$^-$ Sox2$^-$ cells was reduced in the mutant medial FP region (wild-type, 64.0±5.3 cells/section; mutants, 22.2±3.0 cells/section).

Taken together, these results suggest that FP cells in the mesencephalon escape from cell cycle exit, which occurs in cFP cells during the differentiation process, to acquire neurogenic potential, and that *Nato3* activity is required for this suppression of the non-proliferative cFP cell differentiation pathway.

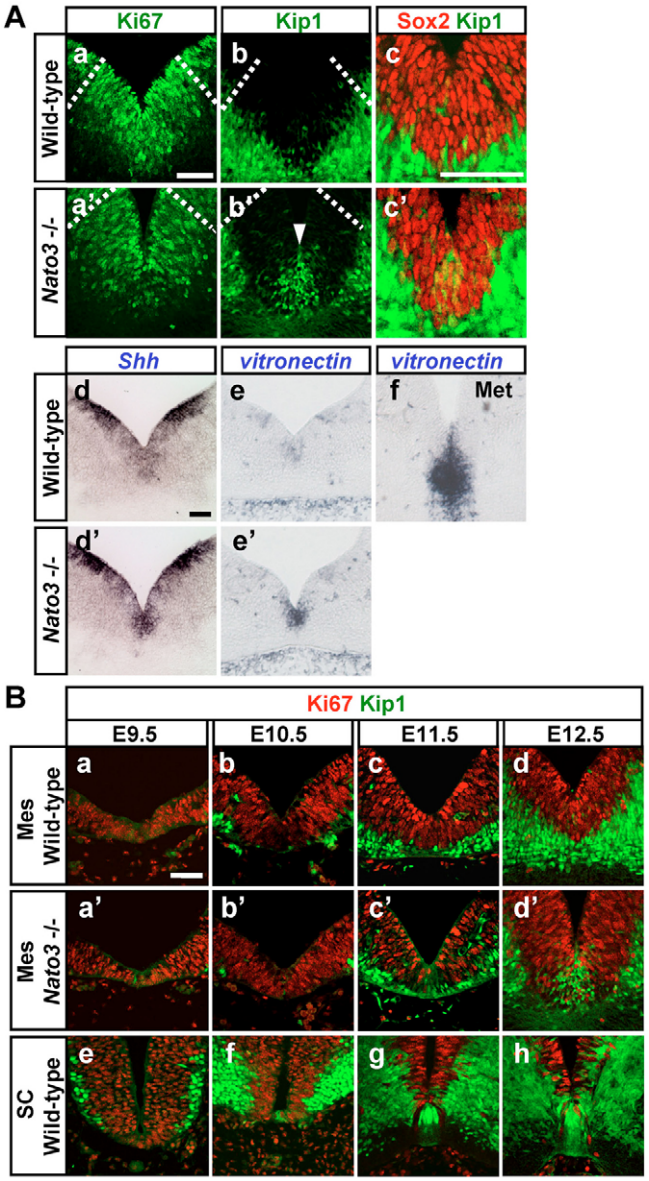

Fig. 5. *Nato3* suppresses non-proliferative cFP-like differentiation in the ventral midline of the mesencephalon. (**A**) Medial mesFP cells exit the cell cycle to differentiate into cFP-like cells in *Nato3* mutants at E12.5. In the absence of *Nato3*, Ki67 expression is downregulated (a′) and p27Kip1 is ectopically upregulated in medial mesFP cells (b′; arrowhead). These cells maintain Sox2 expression (c′) and acquire a high level of expression of *Shh*- (d′) and vitronectin- (e′) like cFP cells in the metencephalon (f). Dotted lines indicate the mesDA/RN domain boundaries defined by Lmx1a expression (data not shown). (**B**) *Nato3*-null ventral midline cells differentiate into non-proliferative cFP-like cells with a time-course similar to that of cFP cell development in wild-type mice. In the wild-type mesencephalon, Ki67$^-$ p27Kip1$^+$ cFP-like cells are not observed during any developmental stage (a-d). In the *Nato3* mutant, FP cells exit the cell cycle from E11.5 onward (a′-d′). In the spinal cord, Ki67$^-$ p27Kip1$^+$ cFP cells appear only after E10.5 (e-h), which is similar to the case with *Nato3*-null mesFP cells. Scale bars: 50 μm.

Nato3 represses Hes1 expression to induce neurogenic differentiation of mesFP cells

We hypothesized that *Nato3* suppresses expression of factor(s) involved in non-dividing cFP cell differentiation to confer neurogenic potential on medial mesFP cells. It has been reported that persistent expression of Hes1 is required for the establishment of boundary cells in the neural tube, including cFP cells, through suppressing neurogenic activity (Baek et al., 2006). We reasoned that Hes1 is involved in the loss of neurogenic activity in the medial mesFP cells induced by the *Nato3* mutation. To this end, we first examined whether Hes1 expression is affected in *Nato3* mutants. In wild-type embryos, Hes1 expression was detected in mesFP cells at E9.5 (Fig. 6Aa). After E10.5, Hes1 expression in the midline was decreased to below the limit of detection (Fig. 6Ab-d). By contrast, in the *Nato3* mutants, downregulation of Hes1 in the midline did not occur and a high level of expression was sustained until E11.5 (Fig. 6Aa′-c′). At E12.5, when most of the medial FP cells in the *Nato3* mutants had exited the cell cycle, the Hes1 level in the medial population declined (Fig. 6Ad′) like in the

cFP cells of wild-type embryos (see Fig. S10 in the supplementary material); however, lateral progenitors still expressed Hes1 at a high level (Fig. 6Ad′). Expression of another *Hes* family member, *Hes5*, was mostly normal in the *Nato3* mutants, although in the medial part of mesFP cells, its expression was rather downregulated (data not shown). These observations suggest that Hes1 is selectively derepressed in mesFP cells because of the loss of *Nato3*. Upregulation of Hes1 was also observed in cFP cells in *Nato3* mutants (data not shown), indicating that Nato3 controls Hes1 expression in FP cells regardless of their anteroposterior location and neurogenic activity. However, even in the presence of *Nato3* in the wild-type condition, Hes1 expression is maintained in cFP cells until E11.5 (see Fig. S10 in the supplementary material), suggesting that Hes1 expression in FP cells might be controlled by other FP factors, as well as by Nato3 (see Discussion).

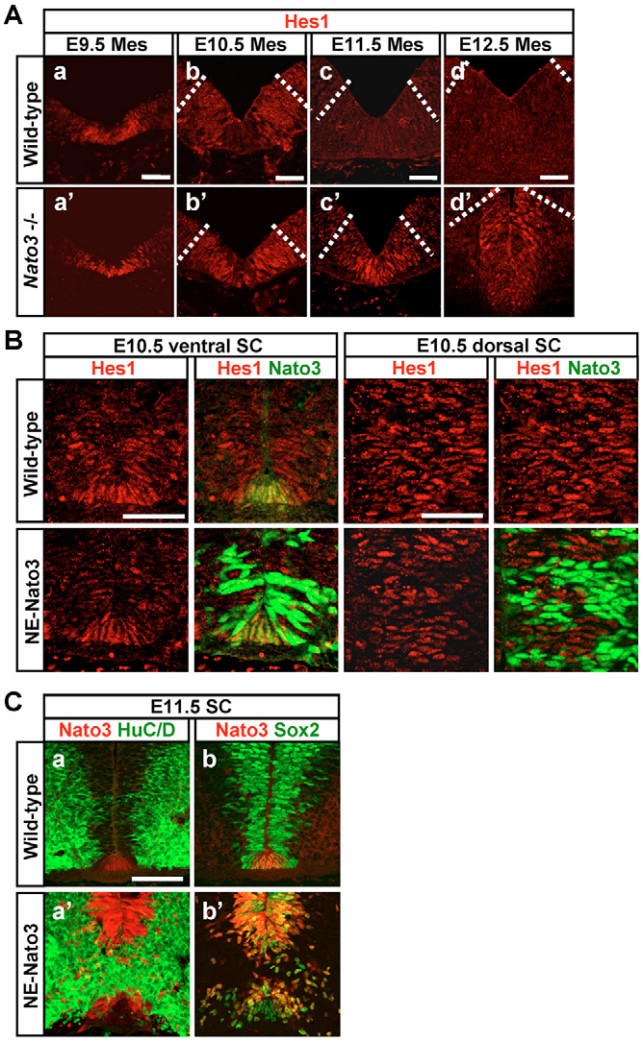

Fig. 6. Nato3 represses Hes1 expression in mesFP cells. (**A**) Hes1 is ectopically induced in the ventral midline of the *Nato3* mutant mesencephalon. In wild-type embryos, a high level of Hes1 expression is detected in mesFP cells at E9.5 (a) but thereafter its expression declines (b-d). In the *Nato3* mutant, Hes1 expression is normal at E9.5 (a′) but its expression in mesFP cells is maintained until later stages (b′,c′). At E12.5, Hes1 expression is decreased in the medial part of mesFP region but is maintained at a high level in the lateral part (d′). Dotted lines indicate the mesDA/RN domain boundaries defined by Lmx1a expression (data not shown). (**B**,**C**) Nato3 represses Hes1 expression in spinal cord neural progenitors. (B) In transgenic embryos expressing *Nato3* under the control of the nestin enhancer (NE-Nato3), Hes1 expression is cell-autonomously repressed at E10.5. (C) As a consequence, premature neuronal differentiation is induced (a,a′) and undifferentiated progenitors are largely eliminated (b,b′) in the ventral spinal cords of NE-Nato3 embryos at E11.5. Scale bars: 50 μm.

To examine whether Nato3 alone can repress Hes1 expression, we generated transgenic embryos expressing *Nato3* under the control of the nestin enhancer (NE-Nato3). Because the Hes1 expression level was low in the developing mesencephalon (data not shown), we analyzed the effect of ectopically expressed *Nato3* in the spinal cord, in which Hes1 expression was readily detectable (Baek et al., 2006). In the spinal cords of NE-Nato3 embryos at E10.5, Hes1 expression in neural progenitors was significantly repressed (Fig. 6B). This effect appeared to be largely cell-autonomous, although some transgene-negative VZ cells lacking Hes1 expression were observed. One day later, consequent neuronal differentiation occurred precociously (Fig. 6Ca,a′) and undifferentiated progenitors were mostly eliminated from the ventral spinal cord (Fig. 6Cb,b′). These results demonstrate that Nato3 is sufficient for repressing Hes1 expression.

We next examined whether overexpression of Nato3 can accelerate neurogenesis in mesFP cells by analyzing NE-Nato3 embryos. In the transgenic mesencephalon, mesDA neuron number was not increased at E12.5 (data not shown). In addition, precocious mesDA generation was not observed at E10.5, the onset of mesDA neurogenesis. These results are consistent with the observation that Hes1 expression was almost completely repressed by Nato3 in wild-type mesFP cells at neurogenesis stages. Thus, the endogenous level of Nato3 expression appears to be sufficient and the rate of mesDA neurogenesis is probably controlled by other factors, such as Otx2 (Omodei et al., 2008), in the presence of Nato3.

Taken together, these observations suggest that Nato3 represses Hes1 to confer neurogenic potential on mesFP cells.

Repression of Hes1 is a prerequisite for proper mesDA generation in mesFP cells

We next asked whether repression of Hes1 by Nato3 in mesFP cells is required for mesDA neurogenesis. In NE-Hes1 embryos expressing *Hes1* under the control of the nestin enhancer, proneural factor expression in mesDA progenitors was significantly decreased (Fig. 7A-B′) and, consequently, generation of mesDA neurons was suppressed (Fig. 7C,C′). In contrast to the *Nato3* mutant phenotype, overexpression of Hes1 led to suppression of proneural gene(s) expression and neurogenesis not only in medial mesFP cells but also in lateral mesFP cells. Similarly, RN neuron generation in the neighboring domain was also suppressed (data not shown). Importantly, p27Kip1$^+$ Sox2$^+$ cells, which resembled the cFP-like cells observed in the ventral midline of the mesencephalon in *Nato3* KO embryos, were observed in the FP domain (Fig. 7D-F′), although Shh expression was not increased in these p27Kip1$^+$ cells and vitronectin and annexin A2 were ectopically induced in all ventral progenitors of NE-Hes1 embryos (data not shown). Although the efficiency of induction of cell-cycle-arrested cFP-like cells by exogenous *Hes1* was not high compared with that brought about by loss of *Nato3*, these results indicate that repression of Hes1 is a prerequisite for proper acquisition of mesDA neurogenic activity in mesFP cells, and that Nato3 acts to confer neurogenic potential on mesFP cells, at least in part, through repressing Hes1.

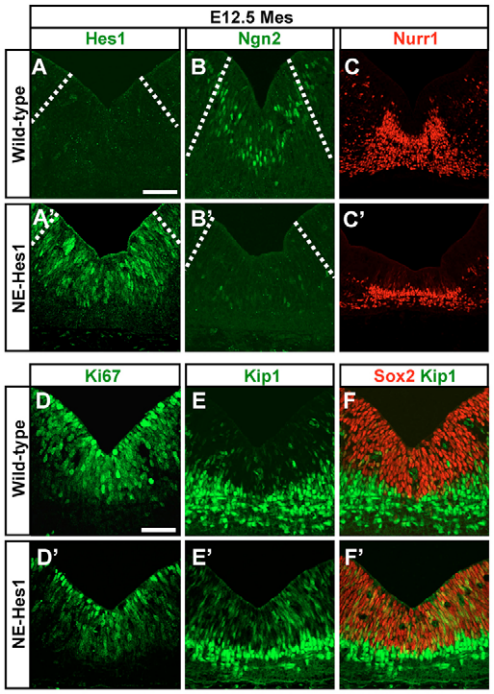

Fig. 7. Forced expression of *Hes1* induces non-neurogenic cFP-like differentiation in mesFP cells. (**A-F'**) Compared with wild-type (A-F), in transgenic embryos expressing *Hes1* under the control of the nestin enhancer (NE-Hes1; A'-F'), proneural factor expression is decreased (B') and, consequently, generation of Nurr1^{+} mesDA neurons is suppressed (C'). Ki67 expression is decreased (D') and p27Kip1 is upregulated (E') in a subset of mesFP cells in NE-Hes1 embryos at E12.5. These cells maintain Sox2 expression (F'). Dotted lines indicate the mesDA/RN domain boundaries defined by Lmx1a expression (data not shown). Scale bars: 50 μm.

Repression of Hes1 by Nato3 is not sufficient for conferring neurogenic potential on medial FP cells

We next asked whether the neurogenic defect observed in medial mesFP cells in the dreher mutant that possesses a loss-of-function mutation in the *Lmx1a* locus (Ono et al., 2007) is caused by non-neurogenic cFP differentiation, as observed in *Nato3* mutants. We first analyzed Nato3 expression in the dreher embryos and observed that Nato3 expression in mesDA progenitors was maintained in dreher mutants, although the level of expression was slightly increased (data not shown), suggesting that Nato3 does not act downstream of Lmx1a to induce neurogenesis in mesFP cells. We next analyzed the growth potential of the mesFP cells in dreher mutants. Although Ngn2 expression was decreased in the medial mesFP cells in the dreher mutants (see Fig. S11a,a′ in the supplementary material), as described previously (Ono et al., 2007), Hes1 and p27Kip1 expression were not detected and Ki67 expression was maintained in the medial FP cells (see Fig. S11b-c′ in the supplementary material) in contrast to the observations in the *Nato3* mutants. Thus, the cause of neurogenic defects in dreher and *Nato3*-null embryos appears to be distinct, and Lmx1a induces proneural gene expression by some mechanism other than controlling Hes1 expression.

Taken together, these results suggest that *Nato3* and *Lmx1a* act on distinct pathways to induce neurogenic activity in mesFP cells. In addition, the maintained Nato3 expression and consequent repression of Hes1 in mesFP cells in dreher mutants indicate that repression of Hes1 is not sufficient for acquiring full neurogenic potential in medial mesFP cells.

DISCUSSION

In the present study, we identified a novel regulator of mesDA neurogenesis, *Nato3*. Loss- and gain-of-function studies demonstrated that *Nato3* confers neurogenic potential on mesFP cells, at least in part, through repressing Hes1. Here we discuss the mechanisms of action of *Nato3* and regulation of FP neurogenesis.

In the developing mesencephalon and caudal neural tube, Nato3 is selectively expressed in FP cells. Although Nato3 is the transcription factor most specifically expressed in FP cells among those identified so far, and the onset of its expression is around the time of FP specification, *Nato3* activity appears to be dispensable for FP cell specification throughout the neural tube. In the caudal neural tube, *Nato3* is essential for proper differentiation of FP cells. Importantly, however, this role was not observed in mesFP cells, suggesting that *Nato3* functions permissively rather than instructively in FP differentiation. Alternatively, the activity of Nato3 could be modified by factors selectively expressed in mesFP or cFP cells. This is in line with another observed role for *Nato3* in conferring neurogenic potential only on mesFP cells. In any case, *Nato3* plays two distinct roles in FP development at different AP locations in the developing neural tube: classical non-neurogenic FP cell maturation in the caudal neural tube and neurogenic FP cell differentiation in the mesencephalon.

Previous reports suggest that in the early stage of development (E9.5), non-neurogenic cFP-like cells exist at the ventral midline of the mesencephalon and Lmx1a-induced Msx1/2 expression induces conversion of FP cells into neurogenic progenitor cells that generate mesDA neurons (Andersson et al., 2006b). However, our present analysis of Ki67 and p27Kip1 expression revealed that non-proliferative cFP-like cells are not detected in the wild-type mesencephalon at any stage. Therefore, mesencephalic ventral midline cells appear to directly differentiate into neurogenic mesFP cells rather than transiently differentiate into cFP-like cells that are converted into neural progenitors. This idea is consistent with the previous observations that Otx2, a master determinant of the mesencephalic identity of FP cells, starts to be expressed before the induction of FP cell fate in the mesencephalon, and that mesFP cells are fated to acquire neurogenic potential by at least E9.75 (Ono et al., 2007).

In the spinal cord, until around E10, medial FP cells remain proliferative but not neurogenic. All medial mesFP cells are proliferative until E11, when they first show proneural gene expression, a sign of neurogenic activity (Ono et al., 2007). Therefore, medial cFP and mesFP cells behave similarly until they start to promote a different differentiation program to become non-proliferative or neurogenic FP cells, respectively. In the absence of *Nato3*, medial mesFP cells start to lose their proliferative property at the stage when they start to show neurogenic activity in the wild-type condition. In this view, *Nato3* probably selects neurogenic differentiation by suppressing the non-neurogenic cFP differentiation program in the context of mesFP cells (Fig. 8A).

It has been reported that *Hes1*, which has a potency to repress proneural gene expression and induce cell cycle arrest, is required for the differentiation of non-neurogenic boundary organizer cells in the developing neural tube (Baek et al., 2006). Despite the fact that FP cells exist in the ventral midline of the mesencephalon, a high level of Hes1 expression was only transiently observed in the early stage of development (E9.5), indicating that sustained Hes1

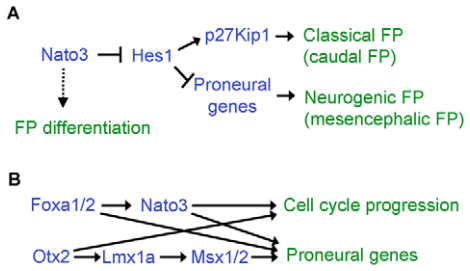

Fig. 8. Current model for the regulation of neurogenic activity in mesFP cells. (**A**) *Nato3* confers neurogenic potential by suppressing differentiation into non-neurogenic cFP cells through repressing Hes1, which suppresses proneural gene expression and induces cell cycle exit. (**B**) Possible transcriptional cascades for the control of cell cycle progression and proneural gene expression in medial mesFP cells. *Nato3* acts downstream of *Foxa2* to induce cell cycle progression (this study). *Otx2* appears to cooperatively regulate this activity (Omodei et al., 2008; Ono et al., 2007; Vernay et al., 2005). *Nato3* also controls proneural gene expression. Otx2 also controls this event through the *Lmx1a–Msx1/2* pathway (Andersson et al., 2006b; Ono et al., 2007). *Foxa2* independently regulates proneural genes (Ferri et al., 2007).

activity is not involved in mesFP cell development. Rather, upregulation of Hes1 was observed in mesFP cells in the *Nato3* mutants that lost neurogenic and growth potential, and forced expression of *Hes1* inhibits neurogenic activity in mesFP cells. Thus, repression of Hes1 is a prerequisite for acquiring a mesFP cell-specific character, specifically, neurogenic potential, and this acquisition requires *Nato3* activity (Fig. 8A). However, loss of *Hes* genes in the spinal cord causes loss of FP cells themselves, instead of conferring neurogenic activity on cFP cells (Baek et al., 2006). Therefore, these observations collectively suggest that transient expression of Hes1 might be involved in the initial specification of FP cell fate and the persistency of Hes1 activity determines the neurogenic potential of FP cells. Then, the question arises as to whether the level of Hes1 expression is a sole determinant of the neurogenic potential in FP cells. In dreher mutants, loss of the mesFP-specific gene *Lmx1a* caused neurogenic defects in medial FP cells in the mesencephalon, even in the presence of *Nato3* activity that consistently represses Hes1. Thus, repression of Hes1 appears to be insufficient for the acquisition of full neurogenic activity by mesFP cells.

Next, the question arises as to whether Nato3 controls neurogenic activity in medial mesFP cells solely by repressing Hes1. This appears not to be the case because forced expression of *Hes1*, even at a higher level compared with that observed in the mesFP cells of the *Nato3* mutant, could not efficiently suppress cell growth in medial mesFP cells. Consistently, despite the fact that derepression of Hes1 occurred in both medial and lateral mesFP cell populations in Nato3 mutants, loss of *Nato3* caused non-neurogenic differentiation of medial mesFP cells but did not suppress neurogenic potential in lateral mesFP cells. In addition, similar levels of growth and neurogenesis suppression were observed in medial and lateral mesFP cells in NE-Hes1 embryos. Therefore, Nato3 appears to regulate not only Hes1 expression but also other pathway(s), which might be selective in the medial aspect of mesFP cells, to control neurogenesis. A recent report proposed that downregulation of Shh by Wnt signaling in FP cells is a determinant of neurogenic activity in mesFP cells (Joksimovic et al., 2009a). Indeed, Shh expression was significantly increased in mesFP cells in the *Nato3* mutant mesencephalon. However, in the wild-type condition, proneural gene induction precedes the onset of Shh downregulation in mesFP cells (Andersson et al., 2006b; Ono et al., 2007). Furthermore, preliminary data showed that sustained Shh expression did not suppress proneural gene expression in mesFP cells or generation of mesDA neurons in vivo (see Fig. S12 in the supplementary material). In addition, Kip1$^+$ cFP-like cells were not induced in the mesencephalon of these NE-Shh transgenic embryos (see Fig. S12 in the supplementary material). This is consistent with the previous observations that loss of Shh in mesFP cells did not affect but rather inhibited mesDA generation (Ferri et al., 2007; Perez-Balaguer et al., 2009). Thus, it is unlikely that upregulation of Shh is a major cause of the neurogenic defect in *Nato3* mutants, although we could not rule out a possible involvement in part.

Our results showed that *Nato3* confers neurogenic potential only on medial mesFP cells. However, Nato3 is also expressed at a high level in non-neurogenic cFP cells, where its activity is required for correct maturation. Indeed, loss of *Nato3* activity causes upregulation of Hes1 in cFP cells and exogenous *Nato3* can also repress Hes1 in spinal neural progenitors. Thus, the Hes1-repressing activity of Nato3 appears to be context-independent. This suggests that the difference in Nato3 activity in controlling neurogenic potential of FP cells is determined by other mesFP- or cFP-selective factors rather than by the transcriptional activity of Nato3 itself.

Several families of transcription factors have been identified as regulators of neurogenic activity in mesFP cells (Andersson et al., 2006b; Ferri et al., 2007; Omodei et al., 2008; Ono et al., 2007; Vernay et al., 2005). The question arises as to whether these factors act on the same pathway determining proneural gene induction. The phenotype caused by loss of these genes appears to be distinct. In dreher mutants, medial mesFP cells did not exit the cell cycle, in contrast to the observations in Nato3 mutants, although both mutants showed similar defects in proneural gene expression. By contrast, the phenotype in *Otx2* cKO mice is highly similar to that in *Nato3* KO mice in terms of the neurogenic and proliferation potential in medial mesFP cells (Omodei et al., 2008; Vernay et al., 2005). This might suggest that the role for *Otx2* in mesFP cells is not only inducing *Lmx1a*, which is required for proneural gene induction (Omodei et al., 2008; Ono et al., 2007), but also controlling other pathways to confer proliferation potential. This idea is in line with the observation that *Otx2*, but not *Lmx1a*, is sufficient to confer mesFP characteristics on cFP cells (Ono et al., 2007). Because Nato3 is expressed throughout FP cells at all AP locations independently of Otx2 expression, Otx2 is unlikely to induce or maintain Nato3 expression in mesFP cells. The fact that the function of Nato3 in cell cycle progression in medial mesFP cells is dependent on mesencephalic context might suggest that Otx2 and Nato3 cooperatively suppress the non-neurogenic cFP differentiation pathway.

Conditional loss of *Foxa1/2* genes causes a significant decrease in neurogenesis in mesDA progenitors (Ferri et al., 2007). Although it has not been reported whether cFP differentiation occurs in these cells, the phenotype is clearly different from that of *Nato3* mutants given that medial and lateral mesFP cells similarly lose proneural gene expression in the *Foxa1/2* mutants. Lateral FP cells in the spinal cord do not have a potency to generate neurons but are still in the Ki67$^+$ proliferative state. This might suggest that neurogenic activity in lateral mesFP cells is regulated at the level of proneural gene expression, independently of the proliferation potential that requires *Nato3* activity, and that *Foxa1/2* controls

proneural gene expression but not cell cycle progression, at least in lateral mesFP cells. Despite the difference in the role for these genes in controlling FP neurogenesis, *Foxa2* appears to act upstream of *Nato3* because ectopic expression of *Foxa2* under the control of the nestin enhancer induced Nato3 expression in the alar and basal plates of the mesencephalon (see Fig. S13 in the supplementary material). Therefore, two possibly cross-talking feed-forward-like pathways, namely the *Foxa1/2–Nato3* and *Otx2–Lmx1a–Msx1/2* pathways, might determine the neurogenic potential in mesFP cells (Fig. 8B). Future detailed analysis will be needed to unmask the transcriptional regulatory cascade underlying AP differentiation of FP cells.

Acknowledgements
We are grateful to Dr T. Imai (KAN Research Institute Inc.) for helpful comments and encouragement. We also thank Dr T. Inoue for critical reading of the manuscript. The monoclonal anti-Shh, anti-En1, anti-Pax6, anti-Msx1/2 and anti-Nkx2.2 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, USA.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042572/-/DC1

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