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Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development

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ABSTRACT

Mesodiencephalic dopaminergic neurons control voluntary movement and reward based behaviours. Their dysfunction can lead to neurological disorders, including Parkinson's disease. These neurons are thought to arise from progenitors in the floor plate of the caudal diencephalon and midbrain. Members of the Foxa family of forkhead/winged helix transcription factor, Foxa1 and Foxa2, have previously been shown to regulate neuronal specification and differentiation of mesodiencephalic progenitors. However, Foxa1 and Foxa2 are also expressed earlier during regional specification of the rostral brain. In this paper, we have examined the early function of Foxa1 and Foxa2 using conditional mutant mice. Our studies show that Foxa1 and Foxa2 positively regulate Lmx1a and Lmx1b expression and inhibit Nkx2.2 expression in mesodiencephalic dopaminergic progenitors. Subsequently, Foxa1 and Foxa2 function cooperatively with Lmx1a and Lmx1b to regulate differentiation of mesodiencephalic dopaminergic neurons. Chromatin immunoprecipitation experiments indicate that *Nkx2.2* and *TH* genes are likely direct targets of Foxa1 and Foxa2 in mesodiencephalic dopaminergic cells in vivo. Foxa1 and Foxa2 also inhibit GABAergic neuron differentiation by repressing the *Helt* gene in the ventral midbrain. Our data therefore provide new insights into the specification and differentiation of mesodiencephalic dopaminergic neurons and identifies Foxa1 and Foxa2 as essential regulators in these processes.

Introduction

Mesodiencephalic (mdDA) dopaminergic neurons control voluntary movement and reward based behaviour and their dysfunction can lead to neurological diseases. These neurons have been extensively studied since loss of mdDA neurons is correlated with Parkinson's disease (Hirsch et al., 1988; Lang and Lozano, 1998). One promising approach for the treatment of Parkinson's disease is cell replacement therapy (reviewed in Lindvall and Bjorklund, 2004; Astradsson et al., 2008). Understanding the genetic network regulating mdDA neuron development will facilitate successful engineering of these neurons from embryonic stem cells, which could then serve as a source of cells for transplantation. mdDA neurons are thought to arise from floor plate progenitors in the caudal diencephalon and midbrain (Marin et al., 2005). Some key determinants of mdDA progenitor specification and differentiation have been identified, including the bicoid class homeobox transcription factor (TF) Otx2 (Puelles et al., 2004; Vernay et al., 2005; Omodei et al., 2008), the LIM homeodomain TF Lmx1a (Andersson et al., 2006b; Ono et al., 2007), the forkhead/winged helix TF Foxa1 and Foxa2 (Ferri et al., 2007; Kittappa et al., 2007) and the proneural basic helix–loop–helix (bHLH) TF Neurogenin2 (Ngn2) (Andersson et al., 2006a; Kele et al., 2006). Lmx1a and Otx2 can induce ectopic dopaminergic neurons in gain-of-function experiments, in a context-dependent manner. Over-expression of Lmx1a by electroporation can induce mdDA neurons only from basal and not alar plate progenitors in chick embryos, suggesting that a ventral determinant must act in concert with Lmx1a to induce mdDA neuronal fate (Andersson et al., 2006b). Similarly, Otx2 can induce ectopic dopaminergic neurons only in the floor plate region of the hindbrain (Ono et al., 2007).

Members of the Foxa subfamily of forkhead/winged helix transcription factors, Foxa1 and Foxa2 (Foxa1/2) are expressed in

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floor plate progenitors as early as embryonic days 8.0 (E8.0) (Ang et al., 1993), making them good candidates to contribute to the specification of mdDA progenitor identity. Previous studies have shown that Foxa1/2 regulate neuronal specification in mdDA progenitors by regulating the expression of proneural bHLH transcription factors, Ngn2 and Mash1 (Ferri et al., 2007). In addition, Foxa1/2 are also required for the expression of Nurr1 in immature mdDA neurons and for the differentiation of mature mdDA neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis (Ferri et al., 2007). However, whether Foxa1/2 play an earlier role in specifying mdDA progenitor identity has not been addressed.

In this paper, we have determined the role of Foxa1/2 in midbrain progenitors by analyzing the phenotype of conditional *Foxa1/2* double mutant embryos. Our results demonstrate that Foxa1/2 positively regulate Lmx1a and Lmx1b (Lmx1a/b) and inhibit expression of the homeodomain TF Nkx2.2 in neural progenitors, resulting in the specification of mdDA progenitor identity. Foxa1/2 bind to upstream regulatory sequences of the *Nkx2.2* and *TH* genes in chromatin immunoprecipitation (ChIP) assays, suggesting a direct regulation of these genes. We also over-expressed Foxa2 and/or Lmx1a into lateral regions of the midbrain of mouse embryos and found that cooperative interactions between these genes are sufficient to induce ectopic dopaminergic neurons. Moreover, Foxa1/2 inhibit GABAergic neuron differentiation in the ventral midbrain by repressing the *Helt*/*Heslike*/*Megane* gene (Guimera et al., 2006; Miyoshi et al., 2004; Nakatani et al., 2007). These results support a model of feedforward and combinatorial interactions of transcription factors in the development of mdDA neurons.

Materials and methods

Generation and genotyping of mutant embryos and animals

All mouse strains were maintained in a mixed MF1-129/SV background. $En1^{KICre/+}$, $Foxa2^{flox/flox}$ and $Foxa1^{loxp/loxp}$ mouse strains were generated as described (Sapir et al., 2004; Hallonet et al., 2002; Gao et al., 2008 respectively). In this paper, we will refer to the $Foxa1^{loxp}$ allele as $Foxa1^{flox}$. $Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ mice were generated by crossing $Foxa2^{flox/flox}$ with $Foxa1^{flox/flox}$ animals. To obtain conditional *Foxa1/2* double mutants, we first crossed $En1^{KICre/+}$ mice with $Foxa2^{flox/flox}$; $Foxa1^{flox/flox}$ animals. Subsequently, $En1^{KICre/+}$;$Foxa1^{fl1ox/+}$;$Foxa2^{flox/+}$ F1 male animals were then mated to $Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ females to generate $En1^{KIcre/+}$;$Foxa2^{flox/flox}$;$Foxa1^{flox/flox}$ double mutants. The $Foxa2^{flox}$ and $Foxa1^{flox}$ alleles were detected by PCR (Hallonet et al., 2002; Gao et al., 2008), whereas the *Cre* transgene was detected by using a pair of primers and PCR conditions as described by Indra et al. (1999). $Shh^{flox/flox}$ (Lewis et al., 2001) animals, also referred to $Shh^{tm2Amc/tm2Amc}$, were purchased from the Jackson Laboratory. $En1^{KIcre/+}$;$Shh^{flox/flox}$ embryos were generated by crossing $En1^{Kicre/+}$;$Shh^{flox/+}$ males with $Shh^{flox/flox}$ females. The Shh^{flox} allele was genotyped by PCR as described on the website of the Jackson Laboratory (http://jaxmice.jax.org). At all times, animals were handled according to the Society of Neuroscience Policy on the Use of Animals in Neuroscience Research, as well as the European Communities Council Directive.

In situ hybridization and immunohistochemistry of brain sections

Embryos were fixed for overnight at 4 °C in 4% paraformaldehyde in 0.1 M PBS and cryoprotected with 30% sucrose in PBS, embedded in OCT compound (VWR International, Poole, UK), and cryosectioned on a cryostat (CM3050S; Leica, Nussloch, Germany). A minimum of three control and three mutant embryos were analyzed, except where indicated. Section in situ hybridization was performed as described previously (Vernay et al., 2005). The following mouse antisense RNA probes have been used: *Gata2* (Nardelli et al., 1999), *Lim1* (Shawlot and Behringer, 1995), *Helt* (Miyoshi et al., 2004), *GAD1* (Behar et al., 1994), Brn3a also known as Pou4f1 — Mouse Genome Informatics (Puelles et al., 2004) and *Islet1* (Puelles et al., 2004).

For immunohistochemistry, sections were incubated overnight at 4 °C with the appropriate primary antibody diluted in 1% BSA in PBS. Sections were then extensively washed in PBS plus 0.1% BSA and incubated 1 h at room temperature with a secondary antibody conjugated with a fluorochrome (Molecular Probes). Sections were then washed and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). The following primary antibodies were used: rabbit anti-Foxa2 (1:1000) (Filosa et al., 1997), goat antiFoxa2 (1:100) (sc-6554, Santa Cruz), guinea-pig anti-Foxa1 (1:500) (Besnard et al., 2004), rabbit anti-Lmx1a (1:1000) (gift from M. German, unpublished), guinea-pig anti-Lmx1b (1:20,000) (gift from T. Müller and C. Birchmeier, Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), mouse anti-Nkx2.2 (1:5) (74.5A5 Developmental Studies Hybridoma Bank), rabbit anti-TH (1:200) (AB152, Chemicon), rabbit anti-Shh (1:100) (sc9024, Santa Cruz), mouse anti-Brn3a (1:100) (sc-8429, Santa Cruz), mouse anti-Islet1 (1:20) (40.2D6, Developmental Studies Hybridoma Bank), rabbit anti-Nurr1 (1:200) (sc990, Santa Cruz), and rabbit anti-Caspase3 active (1:1000, R&D systems). In some cases, staining of nuclei with Toto-3 iodide (1:1000, Molecular Probes) was performed. All images were collected on a Leica TCS SP2 confocal microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

BrdU labeling

Pregnant females were injected intraperitoneally with a solution of BrdU (B-5002, at 10 mg/ml in physiological serum; Sigma) at 100 μg for 1 g of body weight and killed 1 h later. Proliferating cells were revealed by immunohistochemistry with a rat anti-BrdU antibody (1:20, Immunologicals Direct) on frozen sections.

ChIP assays

Mouse E12.5 midbrains were dissected and cross-linked in 1% formaldehyde for 10 min while rotating. Cross-linking was quenched by adding glycine to a final concentration of 0.125 M for 5 min while rotating. The tissue was rinsed in cold PBS and homogenized with a plunger in cold whole cell lysis buffer (10 mM Tris–Cl, pH 8.0, 10 mM NaCl, 3 mM MgCl2, 1% NP-40, 1% SDS) and protease inhibitors. Cells were incubated at 4 °C for 10 min. Lysate was sonicated using the Diagenode Bioruptor for 15 min on high, using 30 s intervals. Cell debris were removed by centrifugation at 13,000 rpm for 10 min, and the supernatant was collected and snap frozen in liquid nitrogen. A 10 μl aliquot of the supernatant was reversely cross-linked by the addition of NaCl to a final concentration of 192 mM, incubated overnight at 65 °C, and purified using a PCR purification kit (Qiagen, CA, USA). The chromatin concentration was determined using a NanoDrop 3.1.0 nucleic acid assay (Agilent Technologies, Santa Clara, CA, USA). 10 μg of chromatin per sample was immunoprecipitated with 2 μg of rabbit anti-Foxa2 (Besnard et al., 2004) or normal rabbit anti-IgG antibody (Millipore #12-370). The immunoprecipitated DNA was analyzed by real-time quantitative PCR (qPCR). Foxa2 binding sites in the *Nkx2.2* and *TH* promoters were determined using position weight matrices from the JASPER database (http://jaspar.cgb.ki.se/).

Real-time qPCR

qPCRs were assembled using Platinum SYBR Green Super mix (Invitrogen). Reactions were performed in triplicates using the ABI 7500 PCR System (ABI). The enrichment of target genes was calculated using the MBP locus as a reference for non-specific DNA, and was calculated by comparing input (sheared genomic DNA) to ChIP material. The ChIP primers for quantitative PCR are as follows: for

real-time qPCR, the following oligonucleotide primers were used: *Nkx2.2* 5′-CCAACAGGAGCGGGACATT-3′ (forward) and 5′-CAAACACAA ATACAAACCGATTGC-3′ (reverse), *TH*: 5′-TGAAGACATCCAAA-AAGCTAGTGAGA-3′ (forward) and 5′-CAAGGGTTCATGTTAG-GAAGGCTATA-3′ (reverse), *Mbp* 5′-GAGCCAGGCTAATGGAGTCAA-3′ (forward) and 5′-AAGCTTCCCCGTTCTGTCACT-3′ (reverse) and *Foxa2* open reading frame gene segment (ORF) 5′-AGCAGAGCCCCAACAA-GATG-3′ (forward) and reverse 5′-TCTGCCGGTAGAAAGGGAAGA-3′.

Electroporation and culture of mouse embryos

Mouse embryos were obtained from pregnant F1 strain mice (CBA/Ca X C57BL/10) at 9.5 days postcoitum (E9.5) and were placed in Tyrode's solution at room temperature. E9.5 embryos were dissected from the uterine walls and freed of decidua capsularis and Reichert's membrane, and the yolk sac was opened. For injection and electroporation, embryos were transferred into the electroporation cuvette between the two platinum plate electrodes (3×8 mm) (CUY520P15, Nepagene, Tokyo, Japan) placed 15 mm apart (Osumi and Inoue, 2001). To target the ventral–lateral side of the midbrain, embryos were oriented and tilted at an angle of approximately 45–60° to the anode in the cuvette. 2–4 μg/μl of plasmid DNA was injected into mesencephalic region of the neural tube using a glass capillary. All the constructs were made in the *pCaggs* vector (Swartz et al., 2001), where gene expression is under the control of the cytomegalovirus immediate-early enhancer and chicken β-actin promoter. Control *pCIG* (Megason and McMahon, 2002) and *pCaggsLmx1a* expression vectors were kindly provided by Drs. A. P. McMahon and Johan Ericson respectively. *pCaggsFoxa2* was made by a cloning a full length *Foxa2* cDNA into the *pCaggs* vector, while the *pCaggsLmx1biresGFP* was made by cloning a full length *Lmx1b* cDNA into *pCIG*. Immediately after injection, five square electrical pulses of 50 V, 50 ms each at 1 s intervals, were delivered through platinum electrodes (CUY520P15) by using a BTX (San Diego) ECM830 electroporator. After electroporation, embryos were transferred into a culture incubator (B.T.C. Engineering, Cambridge, U.K.) and allowed to continue their normal development overnight in 1 ml per embryo of 75% heat inactivated rat serum (produced by NIMR SPF unit) supplemented with Tyrode's solution, and in a continuous-flow atmosphere of 65% O_2, 5% CO_2, and 35% N_2. Next day, the culture medium was changed to 100% heat inactivated rat serum with 95% O_2, 5% CO_2, and continuous-flow atmosphere. The embryos were cultured further under these conditions for 2 more days.

Results

Different requirements for Foxa1/2 and Shh for regional specification of midbrain neural progenitors

Both *Foxa1* and *Foxa2* are expressed in ventral midbrain progenitors as early as E8.5 (Ang et al., 1993). We therefore studied whether these genes are required for specification of mdDA progenitor identity. Because earlier studies have shown that Foxa1 and Foxa2 have similar roles in mdDA neuron development (Ferri et al., 2007), we examined the status of mdDA progenitors in *Foxa1/2* double mutant embryos. Inactivation of Foxa1 and Foxa2 was achieved using the Cre-*loxP* system in mice. Lmx1a is specifically expressed in mdDA progenitors in the ventral midbrain of control embryos (Fig. 1C) and $En1^{KIcre/+}$;$Foxa1^{flox/flox}$;$Foxa2^{flox/flox}$ (referred to as *Foxa1/2cko*) mutant embryos (Fig. 1C′) at E9.5, prior to the complete extinction of Foxa1/2 expression (Figs. 1A, A′, B, B′). However, Lmx1a expression (Fig. 1I, I′) disappeared in *Foxa1/2cko* mutant embryos, concomitant with the loss of Foxa1/2 expression at E9.75 (Figs. 1G–H′). Lmx1b also labels mdDA progenitors, although its expression is broader and includes basal plate progenitors at E9.75 (Fig. 1J, Andersson et al., 2006b). Lmx1b expression was severely reduced in *Foxa1/2cko* mutant embryos at this stage (Fig. 1J′). Identical results were obtained in the midbrain of mutant embryos at E11.5 (Figs. 1M–P, M′–P′). Altogether, these data indicate that Foxa1/2 are required for the maintenance of Lmx1a and Lmx1b expression in mdDA progenitors.

Since Foxa2 regulates Shh expression in the floor plate based on studies in transgenic mice (Epstein et al., 1999), we next examined the expression of Shh and of NKx2.2, a direct target downstream of Shh signalling (Vokes et al., 2007) in the ventral midbrain of *Foxa1/2cko* embryos. Shh expression was present in basal and floor plate progenitors in control embryos at E9.5, E9.75 and E11.5 (Figs. 1E, K, Q) but was greatly reduced at E9.5 (Fig. 1E′), and lost by E9.75 (Fig. 1K′) and at E11.5 (Fig. 1Q′) in the midbrain of *Foxa1/2cko* embryos. In contrast, Nkx2.2 was similarly expressed in control and *Foxa1/2cko* embryos at E9.5 and E9.75 (Figs. 1F, F′ and L, L′ respectively) except for a few cells ectopically expressing Nkx2.2 adjacent to $Lmx1b^{+}$ cells in the ventral midline of mutant embryos at E9.75 (Figs. 1L, L′). By E11.5, Nkx2.2, which is normally restricted to progenitors around the A/B boundary, was ectopically expressed in all ventral progenitors (Figs. 1R, R′). These results indicate that Foxa1/2 are required to maintain Shh expression and inhibit Nkx2.2 expression in the midbrain floor plate.

We next addressed whether loss of Shh in *Foxa1/2cko* embryos could affect the specification of mdDA progenitors. *Shh* null mutant embryos present dorsal–ventral patterning defects in the neural tube (Chiang et al., 1996), and loss of Shh signalling leads to severe reduction in the number of dopaminergic neurons in $En1^{Cre/+}$;$Smo^{flox/-}$ embryos (Blaess et al., 2006). We therefore analyzed the ventral midbrain of $En1^{KIcre/+}$;$Shh^{flox/flox}$ (referred to as *Shhcko*) mutant embryos, where Shh expression is lost in basal and floor plate progenitors at E9.75 and E11.5 (Figs. 2E, E′, K, K′). Interestingly, expression of Lmx1a/b and Nkx2.2 was differentially affected in *Shhcko* embryos compared to the *Foxa1/2cko* embryos. Nkx2.2 expression (Figs. 2F, F′, L, L′) was lost in *Shhcko* at E9.75 and E11.5. As Nkx2.2 is a direct target of Shh and has been shown to require a longer period of Shh signalling than Foxa2 for its induction (Dessaud et al., 2007), we determined whether there was a difference in the timing of inactivation of Shh expression in *Foxa1/2cko* versus *Shhcko* embryos. We analyzed Shh expression at E8.75 (16 somites) in the two different mutants and found that while Shh expression was already lost in *Shhcko* embryo, its expression was only slightly reduced in *Foxa1/2cko* embryos at this stage, suggesting that the earlier loss of Shh signalling likely explains the failure to induce Nkx2.2 expression in *Shhcko* mutant embryos (Supplementary Figs. 1A–B′). On the other hand, loss of Shh signalling does not lead to any changes in the expression of Lmx1a/b (Figs. 2C–D′, I–J′) and Foxa1/2 (Figs. 2A–B′, G–H′) indicating that Shh is no longer required to maintain the expression of these genes in mdDA progenitors in *Shhcko* embryos at E11.5. Shh is therefore required for Nkx2.2 expression, but not for Lmx1a/b or Foxa1/2 expression in the ventral brain from E8.75 onwards. In agreement, Foxa2 expression was also not changed in *Shhcko* compared to control embryos (Supplementary Figs. 1D, D′) and was similarly expressed as Shh in *Foxa1/2* and control embryos (Figs. 1C, C′) at E8.75. Altogether, these results demonstrate different requirements for Shh and Foxa2 in regulating the expression of cell fate determinants in progenitors of the ventral midbrain. Despite maintenance expression of Lmx1a/b in the midbrain, there is a reduction in the number of mdDA neurons at E12.5 and E18.5, likely due to a role of Shh in regulating proliferation of mdDA progenitors in *Shhcko* mutant embryos (Perez-Balaguer et al., 2009).

Foxa2 and Lmx1a/b function cooperatively to specify dopaminergic fate

Since Foxa1/2 are required for Lmx1a/b expression, we also asked whether Foxa2 is sufficient to induce Lmx1a/b expression in the midbrain, using a gain-of-function approach. Experiments were performed with Foxa2 only since Foxa1 and Foxa2 have similar functions in mdDA

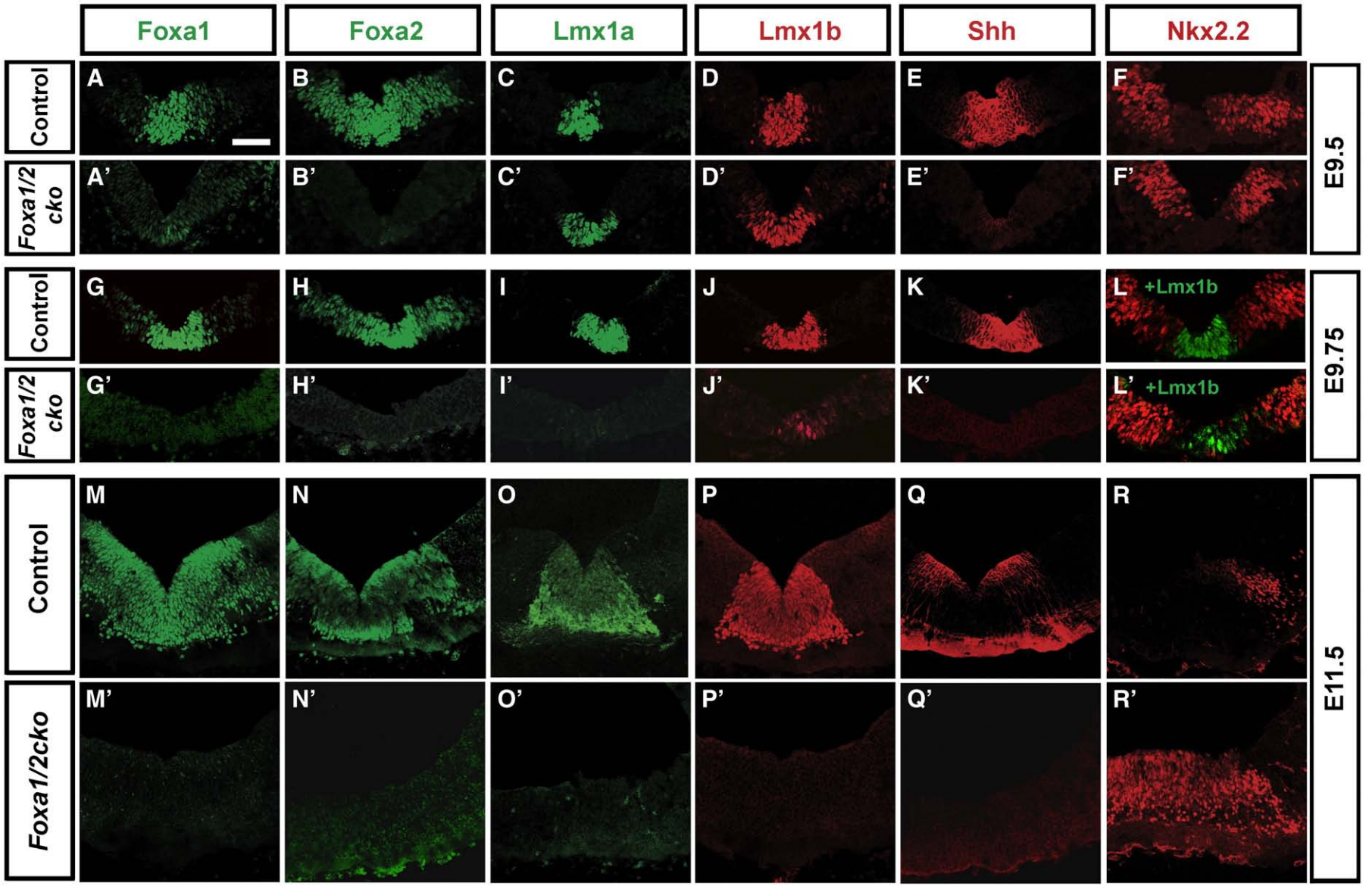

Fig. 1. Foxa1/2 are required to maintain Lmx1a/b and Shh expression and to inhibit Nkx2.2 expression in the midbrain of *Foxa1/2cko* embryos. (A–R, A′–R′) Coronal sections of the midbrain. Only the ventral part of the midbrain is shown. While Foxa1/2 and Shh expression are severely reduced, expression of Lmx1a/b and NKx2.2 are unchanged in Foxa1/2cko compared to control embryos at E9.5 (A–F′). Expression of Lmx1a and Shh is not maintained, while Nkx2.2 expression is expressed in a few cells adjacent to the residual Lmx1b^{+} cells in the floor plate of mutant embryos at E9.75 (L,L′). Massive expansion of Nkx2.2 expression occurs later in the ventral midbrain of mutant embryos at E11.5 (R,R′). Scale bar: 75 μm. Abbreviations: *Foxa1/2cko*, *En1*$^{KIcre/+}$;*Foxa1*$^{flox/flox}$;*Foxa2*$^{f/ox/flox}$.

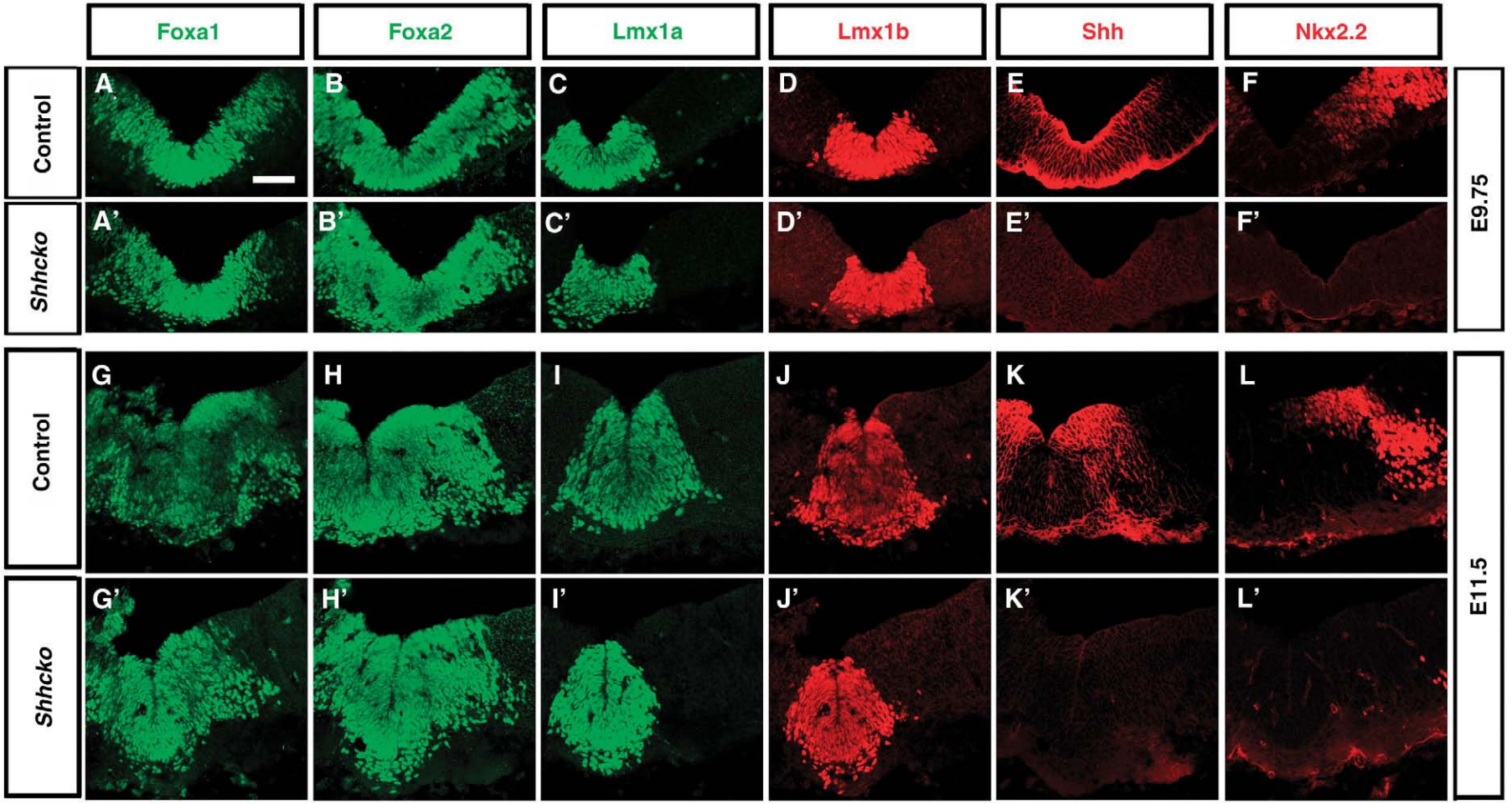

Fig. 2. Shh is required to maintain Nkx2.2 but not Foxa1/2 and Lmx1a/b expression in the midbrain of *Shhcko* embryos. (A–L, A′–L′) Coronal sections of the midbrain. Only the ventral part of the midbrain is shown. Loss of Nkx2.2 expression is observed at E9.75 and E11.5 in mutant embryos. Scale bar: 75 μm. Abbreviations: *Shhcko*, $En1^{KIcre/+}$;$Shh^{flox/flox}$.

progenitors and neurons (Ferri et al., 2007). A Foxa2 expression construct was electroporated into the lateral midbrain of mouse embryos at E9.5 to target progenitors around the A/B boundary. After electroporation, embryos were cultured for three days and then analyzed by immunohistochemistry for expression of mdDA progenitor and differentiation markers. Control electroporation of GFP alone did not perturb the expression of Nurr1, Lmx1b, TH and Pitx3, when comparing the electroporated and non-electroporated sides of the midbrain (Figs. 3A–A″ and data not shown). Nurr1 normally labels immature and mature mdDA neurons adjacent to the floor plate progenitors in the midbrain (Figs. 3A′). Forced expression of Foxa2 was able to induce ectopic Lmx1b$^+$, Nurr1$^+$ neurons in five out of six embryos, albeit in only small numbers of cells. Over-expression of Foxa2 by itself could also induce a few Pitx3$^+$ but not TH$^+$ neurons outside the endogenous mdDA domain (Fig. 3B″). Furthermore, ectopic Lmx1b$^+$, Nurr1$^+$ neurons were generated adjacent to progenitors that expressed Lmx1b (Fig. 3B′) or Lmx1a (Fig. 3B″′) near the alar/basal (A/B) boundary, suggesting that Foxa2 must interact with Lmx1a and/or Lmx1b to induce Nurr1$^+$ neurons in the ventral midbrain.

Previous studies have shown that electroporation of Lmx1a in basal progenitors in chick embryos results in the generation of ectopic mdDA neurons (Andersson et al., 2006b). We examined whether similar results could be obtained in the mouse midbrain. Electroporation of Lmx1a indeed produced supernumerary Lmx1b$^+$, Nurr1$^+$ immature mdDA neurons, but only below the A/B boundary, i.e. adjacent to Foxa2$^+$ basal plate progenitors (Figs. 3C, C′). Electroporation of

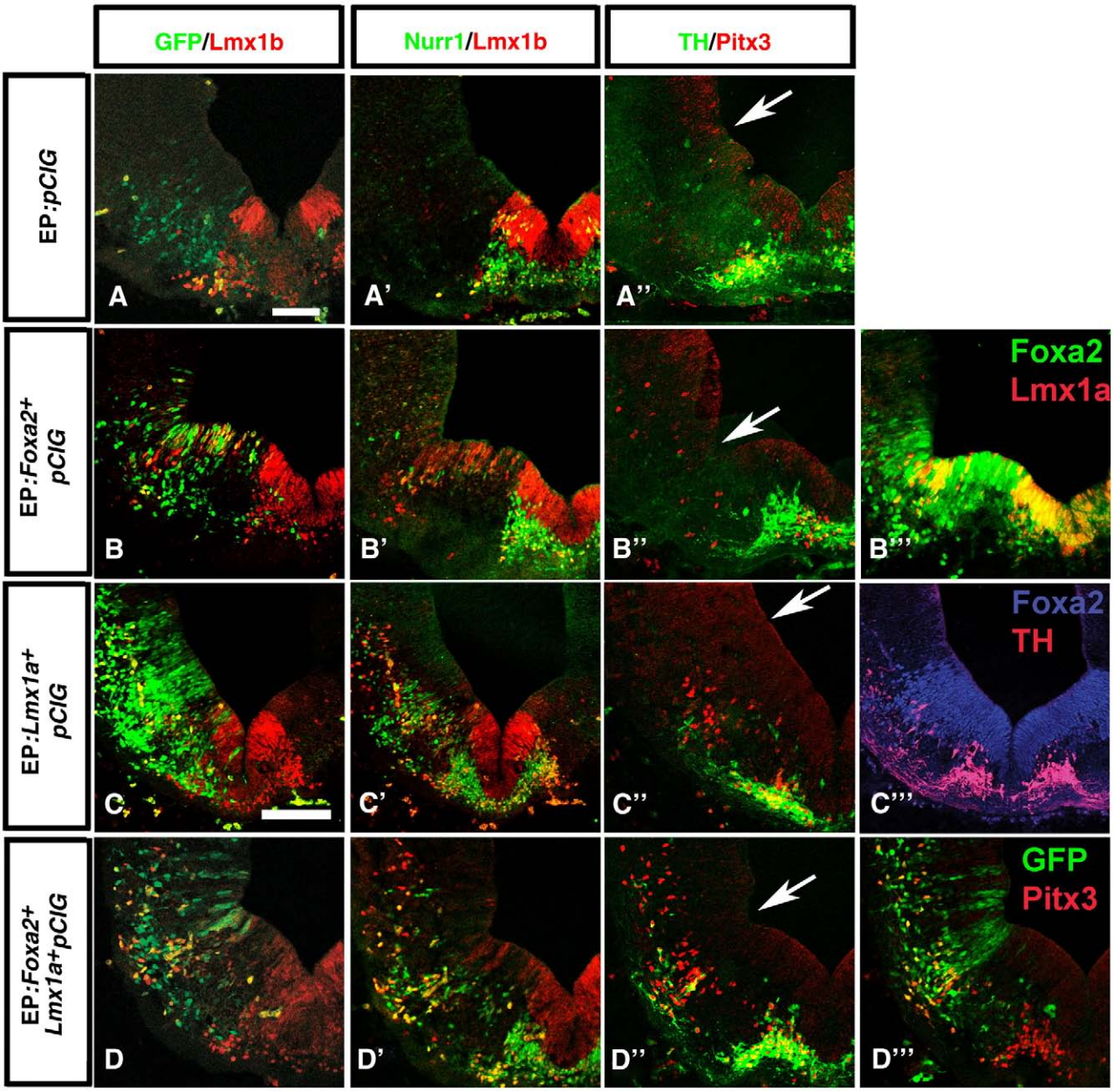

Fig. 3. Foxa2 functions cooperatively with Lmx1a to induce midbrain dopaminergic neurons from dorsolateral midbrain progenitors. (A–D″′) Coronal sections of the midbrain. Only the ventral part of the midbrain focusing on the electroporated side is shown. GFP expression labels electroporated cells in the ventral midbrain (A–D). Normal expression of mdDA immature and mature neuron markers in embryos electroporated with *pCIG* (A–A″). Ectopic mdDA neurons are generated adjacent to progenitors expressing Lmx1a and Foxa2 (B″′) below the A/B boundary. The dorsal limit of Foxa2 expression or the sulcus between the dorsal and ventral midbrain was used to determine the position of the A/B boundary (B″′, C″′ and data not shown). Sparse scattered green cells in B″ correspond to non-specific staining since this expression appears nuclear while TH specific staining is found in the cytoplasm. When both Fox2 and Lmx1a are over-expressed, ectopic mdDA neurons are generated from electroporated GFP$^+$ alar and basal progenitors (D″′). White arrows in A″–D″ indicate the A/B boundary. Scale bar: 75 μm. Abbreviations: EP, electroporation.

Lmx1a alone also generated ectopic Pitx3$^+$, TH$^+$ mature mdDA neurons below the A/B boundary (Fig. 3C″). These results are in agreement with the hypothesis that Lmx1a must cooperate with Foxa1/2 to generate ectopic mdDA neurons.

If Foxa2 and Lmx1a act synergistically to induce ectopic mdDA neurons, then ectopic expression of both genes may lead to the generation of dopaminergic neurons from progenitors in the alar plate as well as basal plate of the midbrain. Consistent with this idea, co-electroporation of Foxa2 and Lmx1a in the midbrain resulted in robust generation of ectopic Lmx1b$^+$, Nurr1$^+$, Pitx3$^+$ and TH$^+$ mdDA neurons in both alar and basal regions. (Figs. 3D–D″′). *pCIG-GFP* was co-electroporated with *Foxa2* and/or *Lmx1a* to trace electroporated cells, and the fact that most ectopic mdDA neurons expressed GFP indicates that these neurons were generated from electroporated cells and not from non-electroporated (GFP$^-$) floor plate progenitors (3D″′ and data not shown). In addition, Lmx1b and the other mdDA neuron markers are mostly co-expressed with GFP in the ectopic mdDA neurons in all electroporated embryos, suggesting that Foxa2 and Lmx1a induce these markers cell autonomously (Figs. 3A–D and data not shown).

Ectopic generation of Nurr1$^+$, TH$^+$ mdDA neurons was also observed when Lmx1b was electroporated alone in basal progenitors (Supplementary Fig. 2). However, since Lmx1b also induced Lmx1a in progenitors (Supplementary Fig. 2), it is not possible to determine whether Lmx1b induces mdDA neurons directly or indirectly through Lmx1a. We favour the hypothesis that Lmx1b acts directly since deletion of Lmx1b in *Lmx1a*$^{dreher/dreher}$;*Shh*$^{Cre/+}$;*Lmx1b*$^{flox/flox}$ embryos results in a more severe loss of mdDA neurons than in *Lmx1a* mutant embryos (C. Yan, R. Johnson, B. Harfe and S.-L. Ang, manuscript in preparation). In summary, data from over-expression studies in mouse embryos indicate that Foxa2 functions cooperatively with Lmx1a and likely also with Lmx1b to coordinate specification and differentiation of mdDA progenitors.

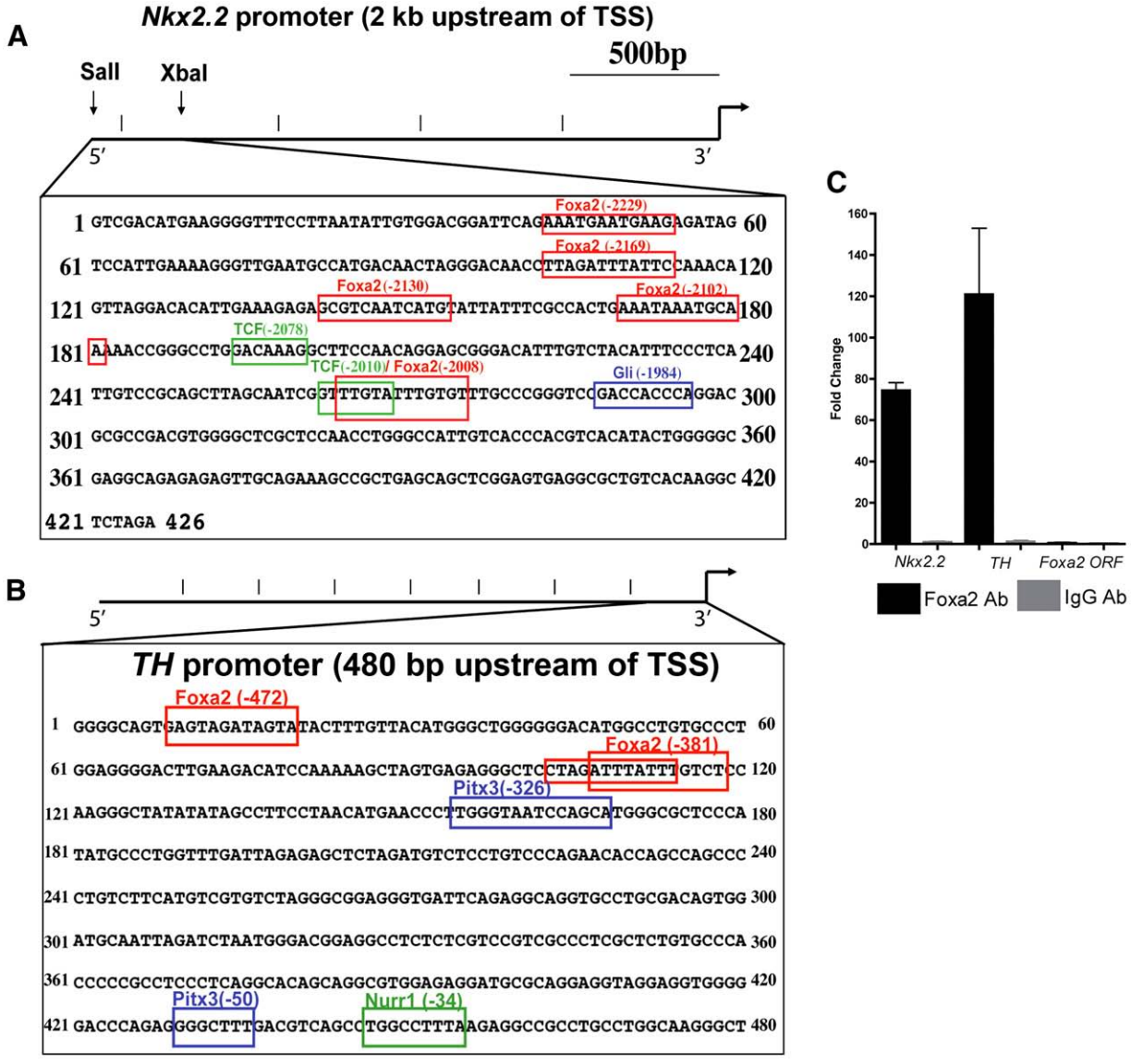

Fig. 4. Foxa2 binds directly to the promoter regions of Nkx2.2 and TH. (A) Scheme of the conserved 426 bp SalI–XbaI promoter region of the *Nkx2.2* gene indicating the positions and conservation of identified and putative TCF (Lei et al., 2006), Gli (Lei et al., 2006; Vokes et al., 2007), and Foxa2 binding sites. (B) Scheme of the proximal region of the mouse TH promoter indicating the positions of identified and putative Pitx3 (Cazorla et al., 2000; Lebel et al., 2001), Nurr1 (Kim et al., 2003) and Foxa2 binding sites. (C) Real-time qPCR from ChIP assays. Significant enrichment is observed for the fragments containing Foxa2 sites in the Nkx2.2 and TH promoter from the Foxa2-ChIP (black bars) and not from IgG-ChIP (grey bars), as compared to a Foxa2 ORF that is used as a control for non-specific binding. Data are presented as the mean ± SEM of triplicate quantifications from at least two immunoprecipitations. Abbreviations: TSS, transcriptional start site.

Foxa2 binds to the promoters of the Nkx2.2 and TH genes in ventral midbrain cells

The loss-and gain-of-function studies described so far and earlier studies (Ferri et al., 2007) indicate multiple and sequential roles of *Foxa1/2* genes in regulating mdDA progenitor specification and differentiation. Potential direct target genes of Foxa1/2 in this lineage include Lmx1a, Nkx2.2, Nurr1 and TH. To better understand the molecular interactions between Foxa1/2 and Nkx2.2 and TH, we performed chromatin immunoprecipitation (ChIP) assays using chromatin prepared from mdDA cells dissected from the ventral midbrain of E12.5 mouse embryos. The ChIP assays were restricted to *Nkx2.2* and *TH*, because promoter regions have already been identified for expression of these genes in the embryonic CNS (Lei et al., 2006) and in dopaminergic neuronal cell lines (Kim et al., 2003), respectively.

Previous studies have shown that a highly conserved 426 bp promoter region of the *Nkx2.2* gene, was sufficient to drive accurate reporter gene expression in the endogenous *Nkx2.2* domains of transgenic mouse embryos (Lei et al., 2006). Five Foxa2 binding sites were found in this element close to two TCF binding sites that are required for reporter gene expression in the p3 domain of the spinal cord (Fig. 4A). Chromatin prepared from microdissected floor plate region of the midbrain of E12.5 embryos was subjected to immunoprecipitation with either Foxa2 antibodies (Foxa2-ChIP) or rabbit immunoglobulin (IgG) antibodies (IgG-ChIP), followed by real-time qPCR. The specificity of the Foxa2 antibodies in the ChIP assay has previously been demonstrated by immunoprecipitation of a known Foxa2 target with chromatin from adult liver cells (Tuteja et al., 2008). The fragment containing putative Foxa2 binding sites in the upstream Nkx2.2 regulatory element was highly enriched in Foxa2-ChIP but not in IgG-ChIP experiments (Fig. 4C). Our results therefore demonstrate in vivo binding of Foxa2 on a Nkx2.2 promoter regulatory region and strongly suggest that Nkx2.2 is a direct target of Foxa2 in the ventral midbrain.

We also analyzed Foxa2 binding to a 1 kb promoter fragment of the *TH* gene containing two putative Foxa binding sites (Fig. 4B). Specific enrichment of this genomic region was also observed in Foxa2-ChIP and not IgG-ChIP experiments, demonstrating that Foxa2 also binds the TH promoter in ventral midbrain cells (Fig. 4C). These in vivo binding data together with results from functional studies in mice strongly suggest that Foxa2 directly inhibits *Nkx2.2* and activates *TH* expression in mdDA progenitors and mature neurons, respectively.

Expansion of GABAergic progenitors and neurons in the ventral midbrain in the absence of Foxa1/2

Midbrain progenitors generate distinct neuronal subtypes at specific dorsal–ventral positions. We examined the consequences of loss of Foxa1/2 activity on the generation of other ventral midbrain neuronal subtypes, besides mdDA neurons. Seven distinct midbrain progenitor domains, referred to as m1–m7, have been proposed to give rise to distinct neuronal subtypes (Ono et al., 2007). The most ventral floor plate progenitors, m7, which express Lmx1a/b, give rise to Pitx3$^+$, TH$^+$ mdDA neurons (Fig. 5A, Ono et al., 2007). Deletion of Foxa1/2 in *Foxa1/2cko* embryos and the subsequent loss of Lmx1a/b expression in progenitors (Figs. 1H′, I′) lead to a complete loss of Pitx3$^+$, TH$^+$ mature mdDA neurons in the ventral midbrain (Fig. 5A′).

Previous studies have shown that the bHLH transcription factor Helt is required for the development of GABAergic neurons in the midbrain (Miyoshi et al., 2004, Guimera et al., 2006; Nakatani et al., 2007). Helt is specifically expressed in m2–m5 progenitor domains that generate GABAergic neurons, and is sufficient to induce supernumerary GABAergic neurons from dorsal progenitors (Miyoshi et al., 2004; Nakatani et al., 2007). Some of these dorsal GABAergic neurons express Nkx2.2 (Nakatani et al., 2007). Since Nkx2.2 was ectopically expressed in neurons near the ventral midline in *Foxa1/2cko* embryos (Fig. 1L′), we examined whether this reflected an increase in the number of GABAergic neurons in these mutants. We found that GAD1, an enzyme involved in the biosynthesis of GABA, was ectopically expressed in *Foxa1/2cko* embryos, in neurons all the way to the ventral midline region (Fig. 5B′), while in control embryos GAD1 expression was restricted to the mantle zone in more dorsal regions (Fig. 5B). Gata2 is required in for GABAergic neuron development (Kala et al., 2008) and Lim1 also marks GAD1$^+$ GABAergic neurons as well as Brn3a$^+$ red nucleus neurons (Nakatani et al., Fig. 5C) in the midbrain. We observed a similar ventral expansion of Gata2 (Figs. 5C, C′) and *Lim1* expression (Figs. 5D, D′), supporting the idea that generation of GABAergic neurons extends to the ventral midbrain in *Foxa1/2cko* embryos. In agreement with this interpretation, expression of Islet1 and Brn3a was missing in oculomotor and red nuclei neurons respectively in *Foxa1/2cko* embryos (Supplementary Fig. 3).

We next examined expression of the determinant of GABAergic progenitors, Helt. *Helt* expression in *Foxa1/2cko* had also expanded in the progenitor domain all the way to the ventral midline (Figs. 5E, E′), indicating that abnormal expression of *Helt* in m6 and m7 progenitors results in the generation of the ectopic GABAergic neurons in the

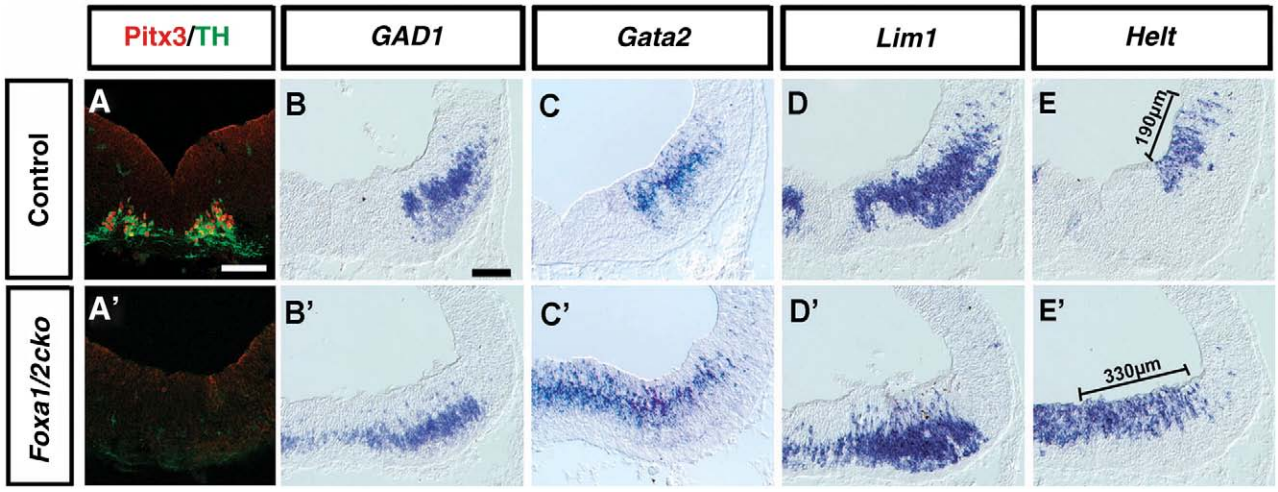

Fig. 5. Expansion of GABAergic neurons at the expense of dopaminergic neurons in the ventral midbrain of *Foxa1/2cko* embryos. Loss of Pitx3$^+$, TH$^+$ neurons occurs coordinately with the expansion of *Helt1*$^+$ progenitors and *GAD1*$^+$, *Gata2*$^+$ and *Lim1*$^+$ GABAergic neurons. *Helt* expression occupies approximately 330 μm and 190 μm of the progenitor zone in one half of the midbrain section of mutant and control embryos respectively. Scale bar: 100 μm. Abbreviations: *Foxa1/2cko*, *En1*$^{Klcre/+}$;*Foxa1*$^{flox/flox}$;*Foxa2*$^{f/ox/flox}$.

midbrain of *Foxa1/2cko* embryos. In support of the notion that the increase in number of GABAergic neurons results from a switch in the fate of ventral progenitors rather than from increased proliferation of GABAergic progenitors, the rate of proliferation as measured by BrdU incorporation after a short BrdU pulse is similar in *Foxa1/2cko* and control embryos at E9.5 (Supplementary Fig. 4). We also determined that there was no change in expression of Otx2 and no increase in cell death in *Foxa1/2cko*, as measured by immunohistochemical staining with rabbit antiCaspase3 active antibodies (Supplementary Fig. 4). Deletion of Foxa1/2 therefore results in re-specification of ventral midbrain progenitors, from an mdDA fate to a GABAergic neuron fate.

Discussion

Foxa1/2 have distinct roles from Shh in regulation of midbrain progenitor identity

In this paper, we have inactivated Foxa1/2 in *En1Cre*;*Foxa1*$^{flox/flox}$;*Foxa2*$^{flox/flox}$ embryos from E9.75 onwards, whereas Foxa2 was deleted in *NestinCre*;*Foxa1*$^{LacZ/LacZ}$;*Foxa2*$^{flox/flox}$ embryos starting at E10.5 in earlier studies (Ferri et al., 2007). These two different conditional *Foxa1/2* mutant mouse models differ only in their timing of inactivation of Foxa1/2 and the present studies led to the identification of an earlier role of Foxa1/2 in regulating neuronal subtype identity in the midbrain. Specifically, Foxa1/2 specify mdDA progenitors by positively regulating Lmx1a/b expression and inhibiting Nkx2.2 and Helt expression. Our loss-of-function studies indicate that Foxa1/2 are required to maintain Lmx1a/b expression, while gain-of-function studies show that Fox1/2 are able to induce Lmx1a/b expression in progenitors, albeit in a context-dependent manner since only basal progenitors can express Lmx1a/b in these experiments. In contrast, Shh is required for Nkx2.2 expression but not for Lmx1a or Lmx1b expression in the ventral midbrain. Our results are therefore consistent with a model whereby *Foxa1/2* are required to maintain *Shh* expression and acts downstream of Shh signalling to inhibit *Nkx2.2* expression (Fig. 6). *Foxa1/2*, but not *Shh* expression in the floor plate, is still required to maintain *Lmx1a/b* and repress *Helt* expression in the ventral midbrain at E9.75. In the absence of Foxa1/2 activity, expansion of the *Helt* expression domain leads to the ectopic generation of GABAergic neurons in the ventral-most region of the midbrain.

Earlier studies have shown that ectopic Nkx2.2 expression in the ventral midbrain prevents the development of red nucleus and DA neurons in *En1*$^{cre/+}$;*Otx2*$^{flox/flox}$ mutant embryos at E12.5 (Prakash et al., 2006). The development of ventral midbrain neuronal subtypes therefore requires repression of Nkx2.2 expression in basal and floor plate progenitors and we show here that Foxa1/2 contribute to this repression. *Nkx2.2* repression is likely direct since Foxa2 binds to a conserved upstream regulatory element in this gene that is required for its expression in the CNS. Earlier studies have also shown that Wnt signals via the Tcf/Lef transcriptional mediator Tcf4, repress *Nkx2.2* expression in the floor plate of the spinal cord (Lei et al., 2006). In contrast, Shh and intracellular transducers of Shh signalling, specifically Gli1 and Gli2 (Gli1/2) transcription factors, are required to positively regulate Nkx2.2 in the ventral midbrain until E8.75 (Figs. 2F, F′ and Blaess et al., 2006). Further studies are required to determine how multiple transcription factors, Gli1/2, Tcf and Foxa1/2, interact to orchestrate the temporal and spatial regulation of *Nkx2.2* expression in the midbrain.

An unresolved issue is how Foxa1/2 regulate Lmx1a/b expression in the floor plate of the midbrain. As the regulatory sequences directing *Lmx1a/b* expression in the midbrain are still unknown, we could not perform ChIP assays to determine whether Foxa2 directly regulates Lmx1a/b. Ectopic Nkx2.2 and *Helt* expression at the ventral midline is unlikely to account for the loss of Lmx1a/b expression, since changes in Nkx2.2 and *Helt* expression occur after Lmx1a/b expression has disappeared. We have also ruled out a contribution of Shh in the maintenance of Lmx1a/b expression in the floor plate. Whether Foxa1/2 regulate Lmx1a/b in a direct or indirect manner remains to be addressed.

It is noteworthy that Foxa1/2 are expressed in the floor plate throughout the anterior–posterior (A–P) axis of the embryos. The restricted expression of Lmx1a in the posterior diencephalon and midbrain floor plate therefore suggests that a spatially restricted transcriptional co-regulator acts in combination with Foxa1/2 to regulate Lmx1a expression. Otx2 is a good candidate to be this rostral determinant since ectopic Otx2 expression is able to induce Lmx1a expression in the hindbrain (Ono et al., 2007). In addition, Otx2 is required for the expression of Lmx1a in the floor plate (Omodei et al., 2008). Taken together, our studies and previous work suggest that Foxa1/2 and Otx2 may function cooperatively to regulate Lmx1a expression in mdDA progenitors.

Sequential combinatorial coding: feedforward mechanism regulating dopaminergic neuron development

Our genetic studies show that Foxa1/2 promote the generation of mdDA neurons through a feedforward loop. Foxa1/2 are sufficient to induce and required for maintaining Lmx1a/b expression in mdDA progenitors. Subsequently, Foxa1/2 function cooperatively with Lmx1a/b to produce mdDA neurons, in part via the regulation of a common

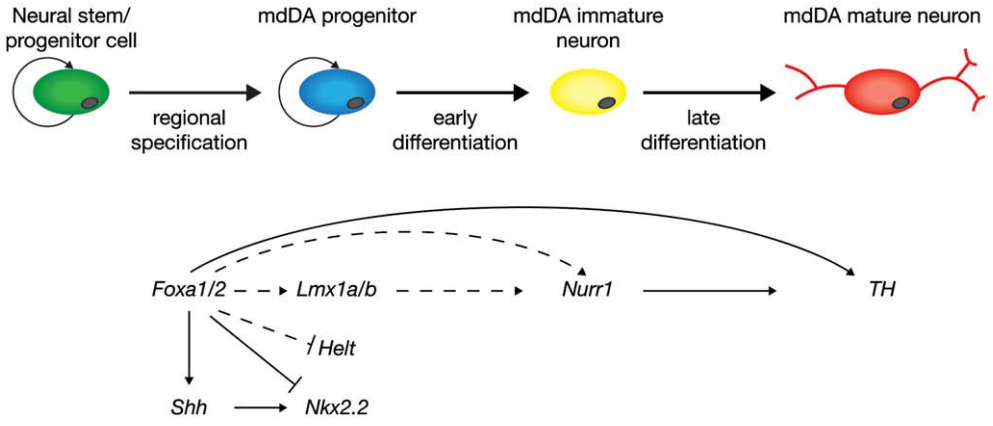

Fig. 6. Combinatorial feedforward model of transcriptional control of midbrain dopaminergic neuron development. The different phases of midbrain dopaminergic neuron development are schematised in the top row. Our results and published work (Ferri et al., 2007; Zetterstrom et al., 1997; Kim et al., 2003) strongly suggest that Foxa1/2 function in a feedforward manner to regulate distinct target genes at different phases by cooperating with sequential downstream transcriptional co-factors. Dotted lines or arrows indicate interactions that may be direct or indirect. Solid lines indicate interactions that have been proven to be direct (Epstein et al., 1999; Vokes et al., 2007) or suggested to be direct based on ChIP experiments.

downstream target, Nurr1, in immature neurons. We have previously shown that Foxa1/2 are required for the initiation of TH expression in mature mdDA neurons (Ferri et al., 2007) and we demonstrate here that Foxa2 binds the TH promoter in embryonic midbrain dopaminergic cells, suggesting that Foxa1/2 directly regulate the TH gene. Interestingly, Nurr1 is also a likely direct regulator of TH expression (Zetterstrom et al., 1997; Kim et al., 2003). It is therefore tempting to speculate that Nurr1 and Foxa1/2 also cooperate to regulate *TH* expression in a subsequent feedforward step during differentiation of immature mdDA neurons. Based on these results, we therefore propose a model whereby *Foxa1/2* function in successive feedforward loops with different co-factors to regulate expression of distinct target genes in immature and mature neurons (Fig. 6). Feedforward regulatory loops have been suggested to provide high fidelity responses during neuronal specification (Baumgardt et al., 2007). Such loops may regulate the fidelity and timing of the successive phases of differentiation in the mdDA lineage, in keeping with our previous demonstration that Foxa1/2 regulate distinct phases of mdDA neuronal differentiation in a dosage-dependent manner (Ferri et al., 2007).

How does Foxa1/2 cooperate with Lmx1a/b to regulate the dopaminergic fate? Lmx1a regulates neurogenesis through activating the expression of Ngn2, as shown in gain-of-function experiments in chick embryos (Andersson et al., 2006b). Foxa1/2 have also been shown to regulate *Ngn2* expression in loss-of-function studies in mice (Ferri et al., 2007). In this paper, we show that Foxa1/2 and Lmx1a/b together induce the expression of Nurr1 in ectopic mdDA neurons in the midbrain using an over-expression approach. Together, these results indicate that Foxa1/2 and Lmx1a/b cooperate to promote mdDA neuron development in part by regulating common targets such as Ngn2 and Nurr1. Foxa1/2 likely also regulate distinct targets from Lmx1a/b, since Shh and Nkx2.2 expression are not affected in *Lmx1a* embryos and $Lmx1a^{dreher/dreher}$;$Shh^{Cre/+}$;$Lmx1b^{flox/flox}$ embryos (Ono et al., 2007; C. Yan, B. Harfe, R. Johnson, S.-L. Ang, manuscript in preparation). Further insights into the mechanisms of cooperation between Foxa1/2 and Lmx1a/b will come from the identification of additional direct target genes of these transcription factors.

In conclusion, we have shown that Foxa1/2 induce mdDA neuron development through a feedforward loop with Lmx1a/b, acting both upstream of and in combination with these factors. Foxa1/2 also have additional roles in midbrain patterning, including maintaining *Shh* expression and inhibiting *Nkx2.2* and *Helt* expression in the ventral midbrain. Foxa1/2 are therefore continuously required during progenitor specification and differentiation of mdDA cells. Foxa1/2 are still expressed in mdDA neurons until adult stage (Kittappa et al., 2007; our unpublished results), and it will be important to determine the later roles of these genes in this lineage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.006.

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