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A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool

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In the developing central nervous system, cellular diversity depends in part on organising signals that establish regionally restricted progenitor domains, each of which produces distinct types of differentiated neurons. However, the mechanisms of neuronal subtype specification within each progenitor domain remain poorly understood. The p2 progenitor domain in the ventral spinal cord gives rise to two interneuron (IN) subtypes, V2a and V2b, which integrate into local neuronal networks that control motor activity and locomotion. Foxn4, a forkhead transcription factor, is expressed in the common progenitors of V2a and V2b INs and is required directly for V2b but not for V2a development. We show here in experiments conducted using mouse and chick that Foxn4 induces expression of delta-like 4 (Dll4) and Mash1 (Ascl1). Dll4 then signals through Notch1 to subdivide the p2 progenitor pool. Foxn4, Mash1 and activated Notch1 trigger the genetic cascade leading to V2b INs, whereas the complementary set of progenitors, without active Notch1, generates V2a INs. Thus, Foxn4 plays a dual role in V2 IN development: (1) by initiating Notch-Delta signalling, it introduces the asymmetry required for development of V2a and V2b INs from their common progenitors; (2) it simultaneously activates the V2b genetic programme.

KEY WORDS: Notch1, Delta-like 4, Foxn4, Gata, Scl (Tal1), Chx10, Spinal cord, Neurogenesis, Chick, Mouse, V2 interneurons, Mash1 (Ascl1)

INTRODUCTION

The neurons and glial cells of the mature central nervous system (CNS) develop from the neuroepithelial progenitor cells that surround the lumen of the embryonic spinal cord and the ventricles of the brain – the so-called ventricular zone (VZ). The spinal cord VZ is a mosaic of progenitor cell domains, each of which generates one or more distinct subtypes of neurons followed by glial cells. The domain pattern is established in response to signals from local organising centres (Briscoe et al., 2000; Ericson et al., 1997). For example, sonic hedgehog diffusing from the notochord and floor plate forms a concentration gradient that specifies five ventral progenitor domains known as p3, pMN, p2, p1 and p0 (ventral to dorsal). This initial patterning phase is followed by the neurogenic phase, during which the progenitor domains give rise to particular combinations of differentiated neurons and glia (Rowitch, 2004). Motor neurons and several types of interneurons (INs) in the ventral spinal cord assemble into local networks that generate the rhythmic output required for locomotion (reviewed by Kiehn, 2006). To understand how locomotor circuits develop, it is necessary to understand the genetic and cellular mechanisms that determine neuron diversity.

The p2 progenitor domain generates two distinct subtypes of INs, V2a and V2b (Karunaratne et al., 2002; Li et al., 2005; Smith et al., 2002; Zhou et al., 2000). Postmitotic V2a INs are characterised by expression of the homeodomain transcription factor Chx10 (Ericson et al., 1997), whereas V2b INs express transcription factors Gata2, Gata3 and Scl (Tal1) (Karunaratne et al., 2002; Muroyama et al., 2005; Smith et al., 2002). How V2 INs incorporate into the local spinal circuitry is not established, although V2a INs are thought to be excitatory (glutamatergic) and to project ipsilaterally (Kiehn, 2006; Kimura et al., 2006). The neurotransmitter phenotype of V2b INs is not known. V2a and V2b INs are derived from common progenitors that initially express the forkhead/winged helix transcription factor Foxn4 (Li et al., 2005) (this paper). How does this homogeneous progenitor pool generate two distinct neuronal subtypes?

The Notch-Delta signalling pathway is often used to establish or to maintain differences between lineally related cells (Artavanis-Tsakonas et al., 1999; Louvi and Artavanis-Tsakonas, 2006). For example, signalling between Notch1 and its ligand delta-like 4 (Dll4) in endothelial cells is necessary for artery-vein discrimination and also for sprouting of lymphatic vessels from veins (Duarte et al., 2004; Seo et al., 2006). We thought it possible that the distinction between V2a and V2b INs might also be established through Notch-Delta signalling. Notch1, 2 and 3 are all expressed in the ventral VZ of the embryonic spinal cord (Lindsell et al., 1996), as are their ligands Dll1, Dll3, Dll4 and jagged 1 (Benedito and Duarte, 2005; Dunwoodie et al., 1997; Lindsell et al., 1996; Mailhos et al., 2001). Unlike Dll1 and Dll3, which are expressed widely throughout the VZ and/or in postmitotic neurons, Dll4 appears to be restricted to the p2 domain of the VZ, suggesting a specific role in V2 IN development (Benedito and Duarte, 2005).

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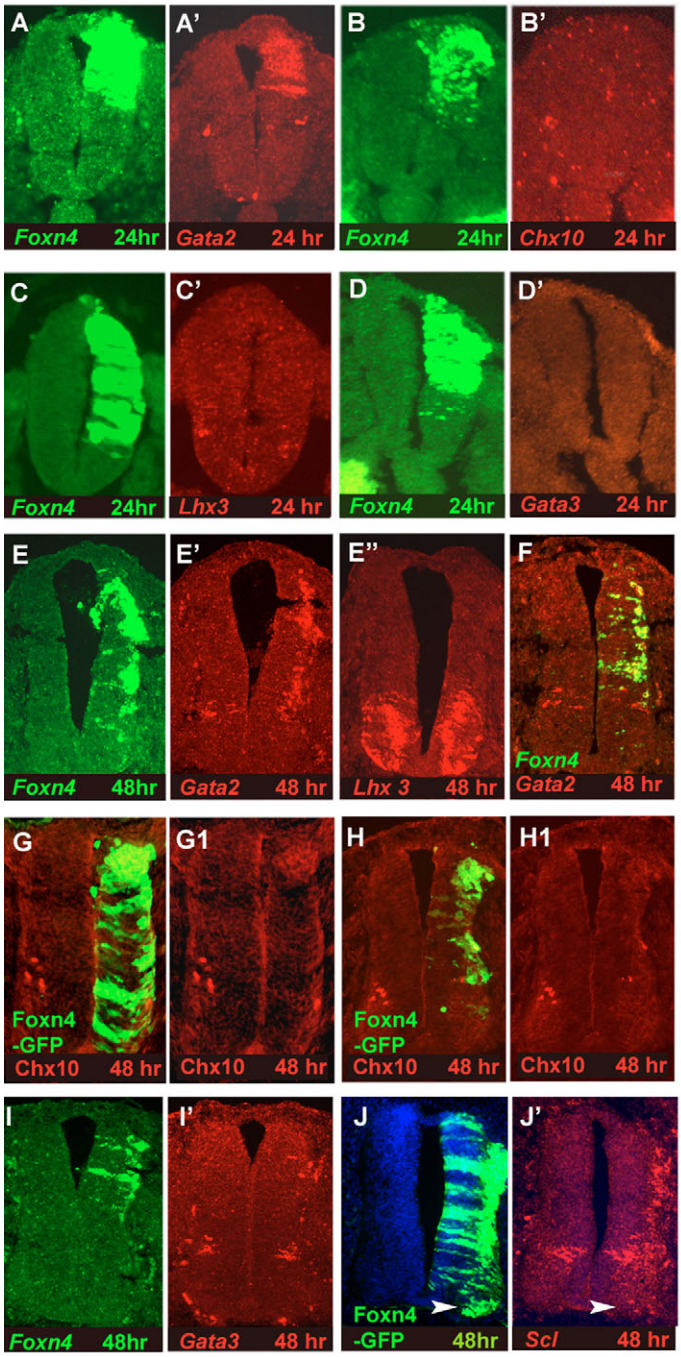

Fig. 1. *Foxn4* is sufficient to induce V2b and suppress V2a interneurons. In this and subsequent figure legends, consecutive sections are labelled A,A′,A″ etc, and different fluorescence channels of the same micrograph are labelled A,A1,A2 etc. (**A-J′**) Chick embryos were electroporated at st12-14 with *β-actin-Foxn4-IRES-GFP* and harvested after 24 or 48 hours. Expression of the vector was confirmed by in situ hybridisation (ISH) for *Foxn4* or immunolabelling for GFP (panels marked Foxn4-GFP). Foxn4 induces robust ectopic expression of *Gata2* at either 24 or 48 hours post-electroporation (A′,E′,F). Foxn4 induced *Gata3* (D′, I′) and *Scl* (J′, note ventral induction, arrowhead) only after 48 hours. Foxn4 does not induce ectopic expression of Chx10 (B′,G,H) or *Lhx3* (C′,E″). On the contrary, Foxn4 represses endogenous Chx10 in the p2 domain (G,H).

We have examined the relationship between Foxn4 and Notch-Delta signalling during development of V2a and V2b sub-lineages. We demonstrated that Foxn4 is a master regulator of the V2b sub-lineage, being necessary and sufficient to induce the V2b determinants Gata2, Gata3 and Scl, while repressing markers of other neuronal lineages. We also found that Foxn4 controls *Dll4* and *Mash1* (*Ascl1*) expression in p2. In gain-of-function assays, Dll4 inhibited the development of V2a INs and, conversely, when Notch1 was conditionally inactivated, V2a INs were overproduced at the expense of V2b INs. Taken together, our data suggest the following model: (1) Foxn4 activates *Dll4* and *Mash1* in common V2a/V2b progenitors; (2) subsequent neighbour-to-neighbour signalling via Dll4 activates Notch1 in a subset of p2 progenitors, which then generate V2b INs under the combined action of Notch1, Foxn4 and Mash1; (3) the complementary set of progenitors fails to activate Notch1 and consequently generates V2a INs.

MATERIALS AND METHODS

Transgenic mice

We used tissue from the following mutant mice: *Foxn4*$^{-/-}$ (Li et al., 2004), *Scl* conditional nulls (Δ*Scl*) (Muroyama et al., 2005), *Notch1* conditional nulls (Yang et al., 2006), *Mash1*$^{-/-}$ (Guillemot et al., 1993). The *Scl* and *Notch1* conditional nulls were crossed to *Nestin-Cre* to eliminate the floxed alleles throughout the CNS.

Electroporation constructs

The complete coding sequence of mouse *Foxn4* was cloned from an E15.5 mouse eye cDNA library by PCR with the primers 5′-CTCCAGGAAATGATAGAAAGTG and 5′-CTGCAGAAGATGGGTAGGTAGAG. The cloned sequence matched the published mouse *Foxn4* mRNA (GenBank accession AY039039), with the exception of nucleotide T288G, which does not change the translated protein sequence. The cDNA (from ATG to stop codon) was cloned into the pCAβ-LINK-IRESeGFPm5-ClaI bi-cistronic expression vector (Schubert and Lumsden, 2005) by PCR.

The *Mash1* vector (gift from Francois Guillemot, National Institute for Medical Research, London, UK) contains the coding sequence of mouse Mash1 under transcriptional control of a synthetic β-actin promoter (*CAGGS*), followed by *IRES-eGFP* (with a nuclear localisation signal).

A human *DLL4* (*hDll4*) expression vector was kindly provided by Ji-Liang Li (John Radcliffe Hospital, University of Oxford, UK). The full-length human *DLL4* coding sequence was PCR-amplified from human placental cDNA, using primers 5′-GGATCCCATATGGCGGCAGCGTCCCGTAGCGCCT and 5′-ACCGGTTCCCGCGGTACCTCCGTGGCAATGACACATTCATTC. *hDll4* was released from the pGEM-T Easy vector (Promega) by *Bam*HI/*Sac*II digestion and inserted into pcDNA3.1/myc-His (Invitrogen).

Electroporation of chick embryos in ovo

Fertilised chicken eggs were incubated at 38°C in a humidified incubator, opened and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were electroporated at st11-16 (Itasaki et al., 1999). The expression constructs [2-5 μg/μl in PBS and 0.8% (w/v) Fast Green] were injected into the lumen of the spinal cord and electroporated using an Intracel TSS20 Ovodyne electroporator with EP21 current amplifier and 0.5 mm diameter home-made platinum electrodes (4-5 pulses of 20-25 volts for 50 milliseconds each).

Tissue preparation and immunohistochemistry

Embryos were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde in PBS. They were then cryo-protected with 20% (w/v) sucrose in PBS, embedded in OCT and frozen for cryo-sectioning (10 μm nominal thickness). The antibodies used were: rabbit polyclonal anti-GFP at 1:8000 (ab290-50, Abcam), rabbit anti-Chx10 at 1:100 (provided by

Thomas Jessell, Columbia University, NY and Connie Cepko, Harvard Medical School, Boston, MA), mouse monoclonal anti-Myc at 1:200 (M4439, Sigma), mouse monoclonal anti-Gata3 at 1:100 (SC268, Santa Cruz), rabbit anti-Olig2 1:8000 (provided by Charles Stiles, Dana Farber Cancer Institute, Boston, MA), mouse monoclonal anti-Hb9 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-gal at 1:2000 (Cappel, ICN Pharmaceuticals), mouse anti-Lim1/2 (Lhx1/5) at 1:30 (DSHB), mouse anti-En1 at 1:5 (DSHB), mouse anti-β-gal (Promega) at 1:300 (with tyramide amplification, Molecular Probes). Some of the sections were incubated with DAPI in PBS in order to visualise cell nuclei before mounting.

In situ hybridisation (ISH)

Our ISH protocols are as described (http://www.ucl.ac.uk/~ucbzwdr/richardson.htm). Some of the templates used to make in situ hybridisation probes were cDNAs obtained by RT-PCR (Invitrogen Kit 11904-018) from a ventral spinal cord chicken cRNA library (Ivanova et al., 2004). PCR forward and reverse primers were as follows: *Foxn4*, 5′-CCCGATGGCTGGAAAAACTC and 5′-AGAGTGTGGAGAGGAGGTGT; *Lhx3*, 5′-AGACGCAGCTGGCCGAGAAGTG and 5′-TGTCCCATGATGCCCAAACC; *Chx10*, 5′-ACAATCTTCACATCCTACCAACTG and 5′-GCTCCATATCTCAAACACCTCAAT; *Gata2*, 5′-TGCCGGCCTCATCTTATCCAC and 5′-TTTGCCATCCCTACATTCTCCTCT; *Gata3*, 5′-AAGCTCTTTCCCCACCCCGACTC and 5′-GGACATCAGACCCATAACCACACG.

A longer chick *Foxn4* template was cloned by RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM-RACE) (Gene Racer Kit), using the supplied 5′ upper primer and 5′-GGCAGAGTGTGGAGAGGAGGTGTC. The cDNA product was 850 bp. The template for chicken *Dll4* was plasmid ChEST714c11 (ARK-Genomics) cut with *Not*I. The mouse *Foxn4* probe includes the ORF minus the first 1000 bp, plus the entire 3′ UTR sequence (Gouge et al., 2001). The *lacZ* probe contained a 3.7 kb *Bam*HI fragment of the *lacZ* gene (*lacZ-pBlueSK*). The mouse *Scl* probe has been described previously (Muroyama et al., 2005).

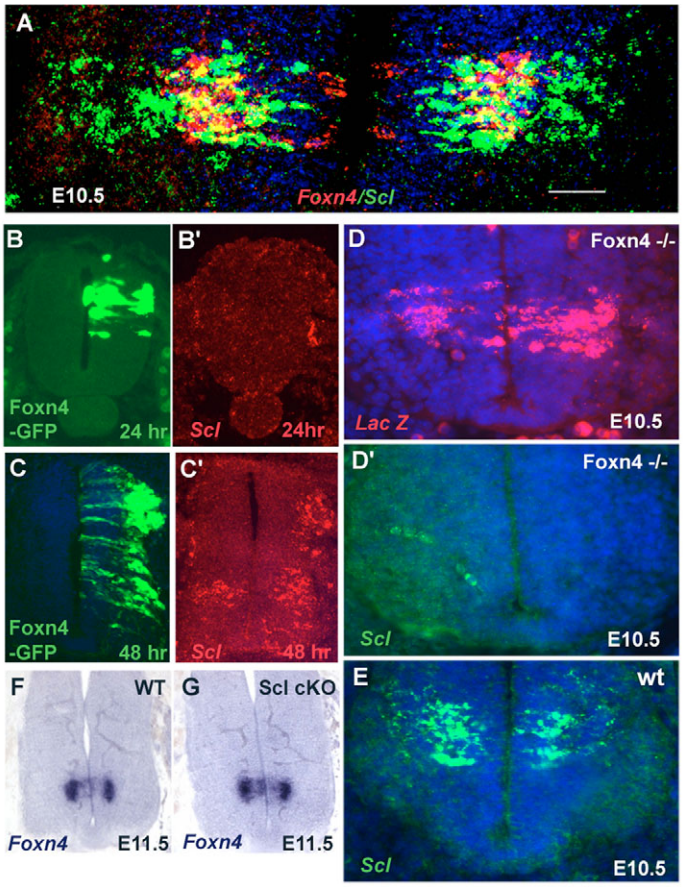

Fig. 2. *Foxn4* lies upstream of *Scl* in V2b interneuron development. (**A**) Double ISH for *Scl* (green) and *Foxn4* (red). Confocal image of wild-type E10.5 mouse spinal cord, showing co-localisation of *Scl* and *Foxn4* in some cells. (**B-C′**) Sections from chicken embryos electroporated at st12-14 with *β-actin-Foxn4-IRES-GFP* and analysed after a further 24 (B) or 48 (C) hours. *Foxn4* does not induce *Scl* after 24 hours (B′). At this stage, endogenous *Scl* is not expressed in the chick neural tube (B′). *Foxn4* does induce ectopic *Scl* after 48 hours (C′). (**D**,**D′**) *Scl* expression in the p2 domain is dependant on functional Foxn4. Consecutive sections from *Foxn4*-null mouse embryos at E10.5 were subjected to ISH for *lacZ* (D) or *Scl* (D′). The row of *Scl*-positive cells visible on the left of this section are endothelial cells. (**E**) *Scl*-positive cells in a wild-type mouse embryo at E10.5. (**F**,**G**) *Foxn4* expression is not dependent on Scl. *Foxn4* expression was visualised by ISH at E11.5 in wild-type (F) and *Scl* conditional null (G) mouse spinal cords (see Materials and methods). Scale bar: 20 μm.

RESULTS

Foxn4 is a master regulator of V2b INs

Foxn4 expression has been described in the developing mouse retina and neural tube (Gouge et al., 2001; Li et al., 2004; Li et al., 2005). In the ventral neural tube it is expressed specifically in the p2 progenitor domain (Li et al., 2005), which generates V2a and V2b INs. We analysed *Foxn4* expression in chick embryos by in situ hybridisation (ISH) during Hamburger-Hamilton stages 10 to 25 (st10-25). We first detected small numbers of *Foxn4*-positive cells in the rostral spinal cord at st13 (see Fig. S1A in the supplementary material). At later stages, the number of *Foxn4*-positive cells increased. As in the mouse, a few cells were present in the VZ close to the lumen, but most accumulated towards the outer margin of the VZ (see Fig. S1B,C in the supplementary material). They are generated exclusively in the p2 progenitor domain, within the region of *Nkx6.1* expression but immediately dorsal to the *Olig2*-expressing pMN domain (see Fig. S1D in the supplementary material and data not shown).

We compared the expression of *Foxn4* with *Chx10*, which marks V2a INs (Ericson et al., 1997), and with *Gata2*, which marks V2b INs (Karunaratne et al., 2002), in chick embryos. There was a significant degree of overlap between *Foxn4* and *Gata2* (see Fig. S1E,G in the supplementary material) but no overlap between *Foxn4* and *Chx10* (see Fig. S1F,H in the supplementary material), implicating Foxn4 in the development of V2b but not V2a INs. In support of this, we found that electroporation of *β-actin-Foxn4-IRES-GFP* into st12-14 chick spinal cord could induce ectopic expression of the V2b markers *Gata2*, *Gata3* and *Scl*, but was unable to induce ectopic V2a markers *Chx10* (0/15 embryos) or *Lhx3* (0/7 embryos) (Fig.1A-F,I,J). *Gata2* was induced robustly by 24 hours post-electroporation (50/50 embryos), whereas *Scl* and *Gata3* required longer (0/5 embryos after 24 hours versus 17/17 embryos after 48 hours for *Scl*; 0/5 embryos after 24 hours versus 6/8 embryos after 48 hours for *Gata3*). The number of Chx10-positive V2a INs generated from the p2 progenitor domain was reduced markedly in these experiments (62±9% reduction, mean±s.e.; 107 cells on the control side versus 33 on the electroporated side; 41 sections from eight embryos) (Fig. 1G,H).

This suggested that Foxn4 might act as a master regulator of the V2b sub-lineage. In a further test of this idea we asked whether Foxn4 can repress alternative IN fates in more-dorsal progenitor domains. We found that electroporated Foxn4 inhibited expression of engrailed 1 (En1), a marker of postmitotic V1 INs (Ericson et al., 1997), and of Lhx1/5, which marks postmitotic INs derived from dorsal progenitor domains dP1-dP6 with the

exception of dP3 (reviewed by Lewis, 2006). A reduction of 31±3% (mean±s.e., *n*=4) was observed for En1 (1173 cells on the control side versus 776 on the electroporated side; 39 sections from four embryos; see Fig. S2A in the supplementary material) and a reduction of 45±13% (*n*=4) for Lhx1/5 (4239 cells on the control side versus 2604 on the electroporated side; 30 sections from four embryos; see Fig. S2B in the supplementary material). These experiments suggest that ectopic expression of Foxn4 can reprogram progenitors to a V2b IN fate.

It has been reported that Scl function is necessary and sufficient for V2b IN development and is required for the maintenance of normal levels of *Gata2* expression (Muroyama et al., 2005). We therefore explored the genetic relationship between *Scl* and *Foxn4*. There was a small but significant overlap between *Foxn4* and *Scl* in wild-type mice (Fig. 2A). *Scl* mRNA expression was abolished in *Foxn4* mutant mouse spinal cords at E10.5 (3/3 embryos) and E11.5 (2/2 embryos) (Fig. 2D,E and data not shown). Conversely, *Foxn4* was expressed as normal in *Scl* conditional null mice (2/2 embryos) (Fig. 2F,G). Also, as described above, *Foxn4* induces *Scl* expression after 48 hours (17/17 embryos) (Fig. 1D, Fig. 2C). Therefore, it seems that *Foxn4* lies upstream of *Scl* in the genetic hierarchy leading to V2b INs.

A negative-control vector with inverted *Foxn4* sequences has been used in parallel with all experiments reported above, without any activity (data not shown). Taken together, our data suggest that *Foxn4* is a master regulator of the V2b sub-lineage. Furthermore, we have shown that Scl lies downstream of Foxn4 in the pathway that governs development of V2b INs.

Foxn4 is expressed in the common progenitors of V2a and V2b INs

It was previously reported that V2a and V2b INs share common, Foxn4-expressing progenitor cells in the VZ (Li et al., 2005). We confirmed this by following expression of β-galactosidase (β-gal) in mouse *Foxn4*$^{+/-}$ heterozygotes, which is possible because the knockout allele contains a functional copy of *lacZ* under *Foxn4* transcriptional control. By double immunohistochemistry we found that β-gal protein was present in cells that co-express Chx10 (Fig. 3A), as well as in cells that express Gata3 (Fig. 3B). By contrast, *Foxn4* transcripts or protein were never found in the same cells as Chx10 or Gata3 (see Fig. S1H in the supplementary material) (Li et al., 2005). The most parsimonious interpretation is that there is a common pool of Foxn4-positive progenitors that generates both V2a and V2b INs. The reason that β-gal can be detected in differentiated V2a as well as V2b INs is presumably because it has a longer half-life than *Foxn4*. In further support of the existence of a common pool of V2a/V2b progenitors, we found that those *Foxn4*-positive cells that lie closest to the lumen (where neural progenitors undergo mitosis) co-express the V2a determinant *Lhx3* (Fig. 3D), as well as *Gata2* (see Fig. 1G in the supplementary material) and *Mash1* (Fig. 3C, Fig. 6A).

Foxn4 activates *delta-like 4* in p2 progenitors

mRNA encoding the Notch ligand delta-like 4 (Dll4) is expressed in scattered cells in mouse and chicken within the p2 progenitor domain (Fig. 4A,B and data not shown). Some of the *Dll4*-positive cells in the p2 domain co-expressed *Foxn4* (Fig. 4A,B). Many of these *Foxn4*/*Dll4* double-positive cells were found at the ventricular surface, where mitosis occurs. Double-positive cells frequently occurred as cell pairs (arrows in Fig. 4B, shown at higher magnification in C,D). These images strongly suggest that *Dll4* and *Foxn4* are co-expressed in cells that are dividing, or in recently separated siblings that are still in contact.

To determine whether *Dll4* and *Foxn4* interact genetically, we performed chick electroporation experiments at st11-12 with *β-actin-Foxn4-IRES-GFP*. Foxn4 induced ectopic expression of *Dll4* at 34 hours post-electroporation in 12/12 embryos analysed (Fig. 4F). A control vector with inverted *Foxn4* sequences had no such effect (data not shown). Consistent with these observations, *Dll4* expression was abolished in the p2 domain of *Foxn4*-null mice at E10.5 (3/3 embryos analysed) and E11.5 (2/2 embryos analysed) (Fig. 4E and data not shown). We conclude that Foxn4 is necessary and sufficient for activation of *Dll4* in p2 progenitors.

Dll4 inhibits V2a lineage progression

To discover whether *Dll4* is involved in the specification of V2 INs – possibly through its interactions with Notch – we performed gain-of-function experiments in chick neural tube by electroporating an expression vector encoding human *DLL4* (CMV-hDll4-Myc). We performed two sets of experiments. In the first, we electroporated at st11-13 and analysed the embryos after a further 44 hours (st19-20). In 16 embryos analysed, we found no ectopic induction of Chx10 immunoreactivity or *Gata2* mRNA. By contrast, a reduction of Chx10 and *Gata2* expression was observed on the electroporated versus the control side, Chx10 being more strongly repressed (~80% reduction) than *Gata2* (~35% reduction) (63 Chx10-positive cells on the control side versus 12 on the electroporated side, compared with 90 *Gata2*-positive cells on the control side versus 58 on the electroporated side; 24 sections from four embryos; data not shown). In the second set of experiments, we electroporated at st14-16 and analysed the embryos after a further 48 hours (st21-23). In this set of experiments, 15 embryos were analysed for Chx10 immunoreactivity and *Chx10*, *Scl* and *Gata2* mRNA (Fig. 5). As in the first experiment,

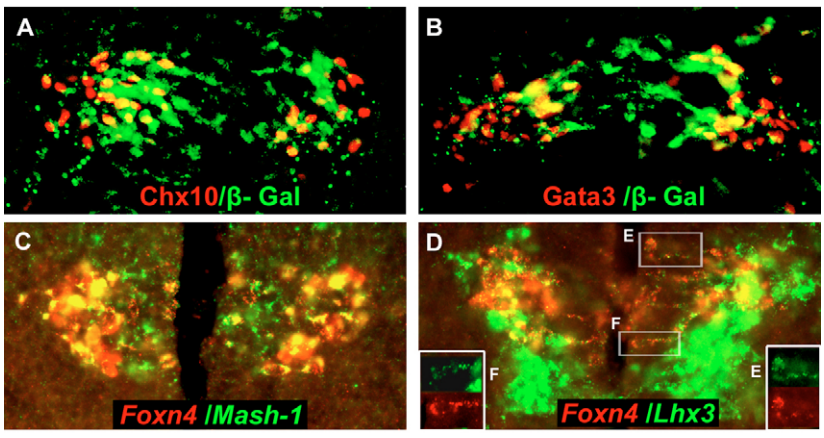

Fig. 3. *Foxn4* is expressed in common precursors of V2a and V2b interneurons. (**A**,**B**) *Foxn4*$^{+/-}$ mouse embryos were labelled by double immunohistochemistry for β-galactosidase (β-gal, green) and either Chx10 or Gata3 (red). Confocal microscopy reveals cells that are double labelled for β-gal and either Chx10 (A) or Gata3 (B), suggesting that Foxn4-expressing progenitors give rise to both V2a and V2b interneurons (INs). (**C-F**) Consistent with this conclusion, *Foxn4*-positive progenitors co-express *Mash1* (C) and *Lhx3* (D), markers that later segregate into V2b and V2a INs respectively. Individual *Foxn4*/*Lhx3* double-positive cells (boxes E and F) are reproduced, with fluorescence channels separated, in the lower left and lower right corners, respectively, of D.

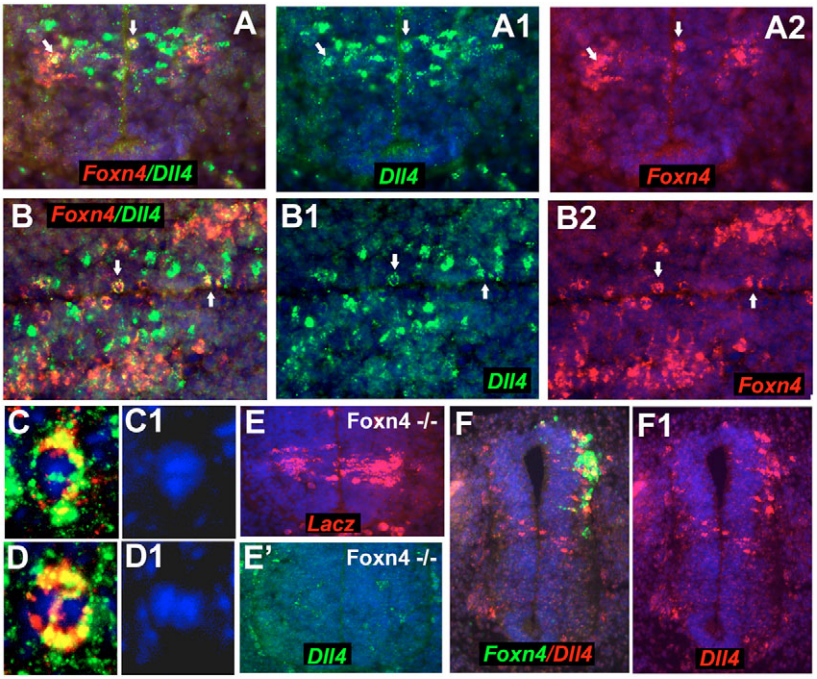

Fig. 4. *Foxn4* is necessary and sufficient to induce *Dll4* in the p2 domain. (**A-D1**) Double ISH for *Dll4* (green) and *Foxn4* (red) in wild-type E10.5 mouse embryos, counter-stained with Hoechst to visualise cell nuclei. A and B are transverse and longitudinal sections, respectively, of spinal cord. *Foxn4* is expressed in some of the *Dll4*-positive cells within and outside the VZ (arrows). A significant proportion of double-labelled cells at the ventricular surface are pairs of cells in contact with each other, presumptive daughters of a recent progenitor cell division (e.g. arrows in B). Examples of these are shown at higher magnification in C,D; note the paired nuclei in C1,D1. (**E**,**E'**) *Foxn4*-null mouse embryos at E10.5. (E) *lacZ* expression under *Foxn4* control. (E′) *Dll4* expression in the p2 domain is abolished. (**F**,**F1**) Double ISH for *Foxn4* (green) and *Dll4* (red) showing that Foxn4 induces ectopic expression of *Dll4* in electroporated st11-13 chick neural tube.

there was no ectopic expression of Chx10 protein or mRNA but a strong repression of Chx10 protein on the electroporated versus control side (51±5% reduction, mean±s.e.; 137 sections from 13 embryos; two-tail *t*-test=3.6 at *P*=0.001) (Fig. 5A,B). *Gata2* mRNA was expressed ectopically in some embryos (19/63 sections in five out of 15 embryos). In general, the induction of *Gata2* was modest and always restricted to the p1-p0 domain (Fig. 5D′, white arrow). Despite this small amount of ectopic expression, the total amount of *Gata2* signal (estimated by counting pixels with ImageJ) was not detectably different on the electroporated versus control sides (594±83 versus 562±83 pixels, respectively; 80 sections from 7 embryos; two-tail *t*-test=0.3 at *P*=0.8, not significant) (Fig. 5D′,E). The *Scl* signal was also not significantly different between electroporated and control sides (396±64 pixels versus 361±57, respectively; 86 sections from 9 embryos; two-tail *t*-test=0.5 at *P*=0.6, not significant), nor was there any ectopic expression of *Scl* (Fig. 5D″,E). These results suggest that at st14-16, Dll4 overexpression specifically represses the V2a fate with little or no effect on V2b fate. In *Dll4* electroporations, some cells were Dll4-Myc/Chx10 double positive (Fig. 5C), indicating that expression of Dll4 is compatible with expression of Chx10 in the same cell.

Foxn4 induces *Mash1* in the p2 domain

The extensive overlap of *Mash1* and *Foxn4* expression in the mouse p2 domain (Fig. 3C, Fig. 6A) suggested some form of regulatory relationship. We therefore explored the interactions between *Foxn4* and *Mash1* in more detail. We confirmed the finding of Li et al. (Li et al., 2005) that *Foxn4* is expressed as normal in *Mash1*-null spinal cord (Fig. 6C,D). After electroporating *β-actin-Foxn4-IRES-GFP* in the chick spinal cord at st13-14, we found strong ectopic induction of *Cash1* (the chick homologue of *Mash1*) after 24 hours (6/6 embryos) and 48 hours (3/5 embryos; Fig. 6B and data not shown). The negative control vector with inverted *Foxn4* sequences had no activity (data not shown). These experiments indicate that *Foxn4* is upstream of and controls expression of *Mash1* in p2, and fits with the observation that *Mash1* expression in p2 is lost in *Foxn4*-null mice (Li et al., 2005).

Mash1 stimulates *Dll4* expression but does not induce V2b INs

Mash1 controls the expression of *Dll1* in the ventral telencephalon and dorsal spinal cord (Casarosa et al., 1999), so we asked whether Mash1 can also induce *Dll4*. We electroporated full-length mouse *Mash1* (*β-actin-Mash1-IRES-GFP*) into st13-14 chick neural tube. After 24 hours of incubation, 8/8 embryos showed clear ectopic induction of *Dll4* on the electroporated side (Fig. 6E). After 48 hours, 5/5 embryos displayed weaker but still clear induction of *Dll4* (data not shown). In none of the 13 embryos analysed did we find any ectopic expression of *Chx10*, *Gata2* or *Scl* transcripts or Chx10 immunoreactivity (Fig. 6F,G,H′,H″ and data not shown). On the other hand, we observed a loss of endogenous Chx10-positive INs in the p2 domain of 5/5 embryos analysed (76±6% reduction, *n*=23) (Fig. 6F,F′,G), with little or no concomitant reduction of *Gata2* or *Scl* (Fig. 6H′,H″). These data suggest that induction of *Dll4* and consequent repression of Chx10-positive V2a INs by Foxn4 might be mediated indirectly via Mash1. However, we found that *Dll4* is expressed as normal at E10.5-11 in *Mash1*-null embryos (4/4 embryos; Fig. 6I,J). Therefore, Mash1 might be involved in maintaining or reinforcing *Dll4* expression but is not required for its initiation. Although Mash1 is necessary to develop the V2b fate (Li et al., 2005), it is not sufficient to do so, judging by its inability to induce ectopic *Gata2* or *Scl* expression. Therefore, it appears that the V2b program of gene expression is absolutely dependent on Foxn4.

Notch1 is required for generation of V2b INs

The fact that Dll4 preferentially represses the V2a fate suggests that the Notch-Delta system might be responsible for the V2a-V2b binary fate decision in p2 progenitors. To test this, we analysed *Notch1* mutant (cKO) mouse embryos at E10.5 and E11.5 by ISH for *Foxn4* or *Scl*, or by immunohistochemistry for Chx10, Gata3, Olig2 or Hb9 (Hlxb9) (Fig. 7). Olig2 is a basic helix-loop-helix transcription factor that is expressed in the progenitors of motor neurons (MNs) and oligodendrocytes but not in postmitotic MNs (Lu et al., 2000), whereas Hb9 is a transcription factor expressed in early committed

MNs (Thaler et al., 1999). At E11.5, no Gata3 (0 versus 99±3, *n*=14 sections from three embryos; two-tail *t*-test=26, *P*<0.001) or *Scl*-positive cells were present in the ventral spinal cord of *Notch1* cKO mice (3/3 embryos analysed) (Fig. 7A-D,M-N). Instead, twice the normal number of Chx10-positive cells was observed (200±10 versus 102±3, *n*=16 sections from three embryos; two-tail *t*-test=8.6 at *P*<0.001) (Fig. 7A-D), as previously reported (Yang et al., 2006). pMN progenitors that express Olig2 were drastically reduced at this age (2±0.5 versus 39±2, *n*=14 sections from three embryos; two-tail *t*-test=17, *P*<0.001), but the number of Hb9-positive cells was not significantly affected in the *Notch1* mutant (3/3 embryos analysed) by comparison with wild-type mice (175±16 versus 165±8, *n*=14 sections from three embryos; two-tail *t*-test=0.6, *P*=0.6) (Fig. 7D,E-F). It seems that Notch1 signalling is necessary to prevent premature differentiation of most progenitor cells in the ventral cord, judging by the loss of the ventral VZ in the mutant (Yang et al., 2006). However, loss of Notch1 does not seem to result in respecification of pMN progenitors to p2 progenitors, as originally proposed (Yang et al., 2006). Rather, the phenotype is more consistent with respecification of V2b to V2a INs, consistent with the idea that signalling through Notch1 is required for V2b IN development.

Foxn4 is very much reduced in the E11.5 *Notch1* conditional null spinal cord (Fig. 7I,J, arrow). A simple interpretation is that the *Foxn4*-positive progenitors of V2a and V2b INs differentiate prematurely and completely into V2a INs in the mutant and, in doing

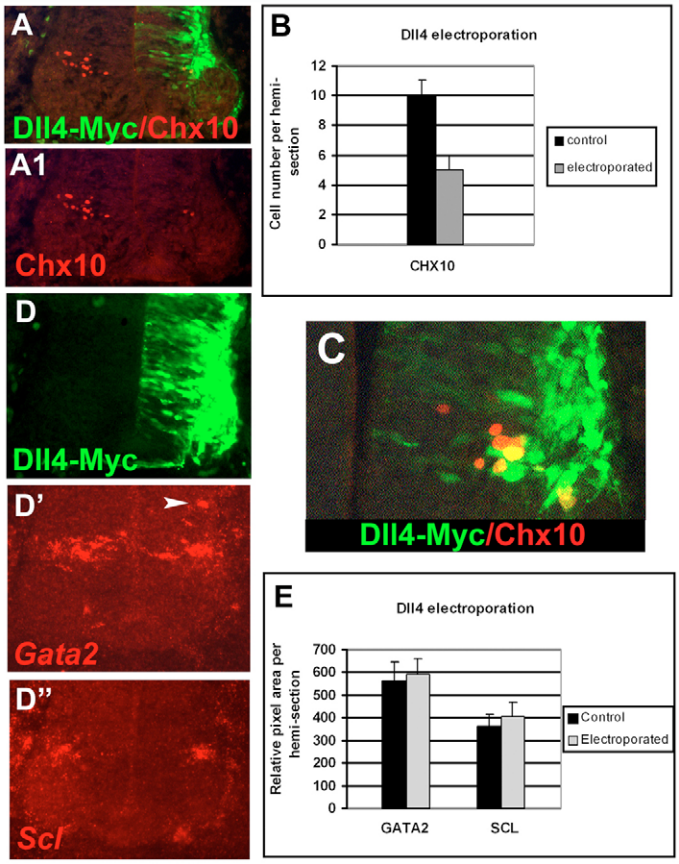

Fig. 5. Dll4 inhibits V2a lineage progression. Chick embryos were electroporated with human *DLL4* (*hDll4*) in the form of *hDll4-Myc* at st14-16 and analysed after 48 hours. (**A-B**) Double immunolabelling for Chx10 (red) and hDll4-Myc (green) showing repression of Chx10-positive cells. (B) Quantification of Chx10-labelled cells showed a ~50% decrease on the *hDll4*-electroporated side compared with the contralateral, control side. (**C**) Some *hDll4*-electroporated cells co-express Chx10, consistent with the idea that Dll4 can suppress V2a generation in a non-cell-autonomous fashion. (**D**) Immunolabelling for hDll4-Myc (green). (**D′**) Dll4 exceptionally can induce *Gata2* (arrowhead). (**D″**) Dll4 does not affect *Scl* expression. (**E**) *Dll4* does not greatly affect generation of V2b INs, judging by ISH. Quantification of V2b markers *Gata2* and *Scl* by pixel-counting software showed no significant effect on V2b production (see text for statistics).

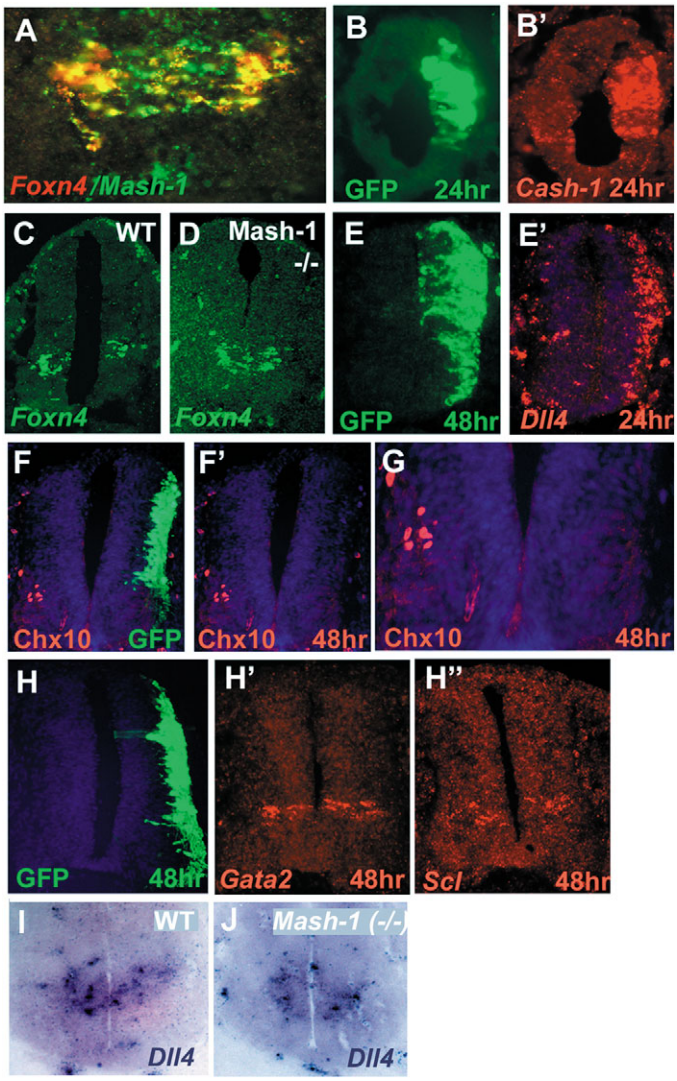

Fig. 6. Foxn4 controls *Mash*1 expression. (**A**) Double ISH for *Foxn4* (red) and *Mash1* (green) in E10.5 mouse cord. Note the extensive overlap in the p2 domain. (**B**,**B′**) Foxn4 induces ectopic expression of *Cash1* in chick electroporation experiments. (**C**,**D**) *Foxn4* expression does not depend on *Mash1*; there is no noticeable change in the *Foxn4* ISH signal in *Mash1*-null mice compared with wild type. (**E**,**E′**) Electroporation of *β-actin-Mash1-IRES-GFP* in the st13-14 chick neural tube induces *Dll4* after 24 hours. Mash1 expression was confirmed by GFP immunolabelling (E) and *Dll4* by ISH (E′). (**F-G**) Mash1 did not induce ectopic Chx10, but repressed endogenous Chx10 V2a INs in the p2 domain. G is a magnified view of the ventral part of panel F′. (**H-H″**) Also, Mash1 did not induce ectopic V2b markers *Gata2* or *Scl*. (**I**,**J**) Despite the fact that Mash1 is sufficient to induce *Dll4* in chick (see E,E′), Mash1 is not required for *Dll4* expression in mice; *Dll4* is expressed as normal in the ventral spinal cord of *Mash1*-null mice.

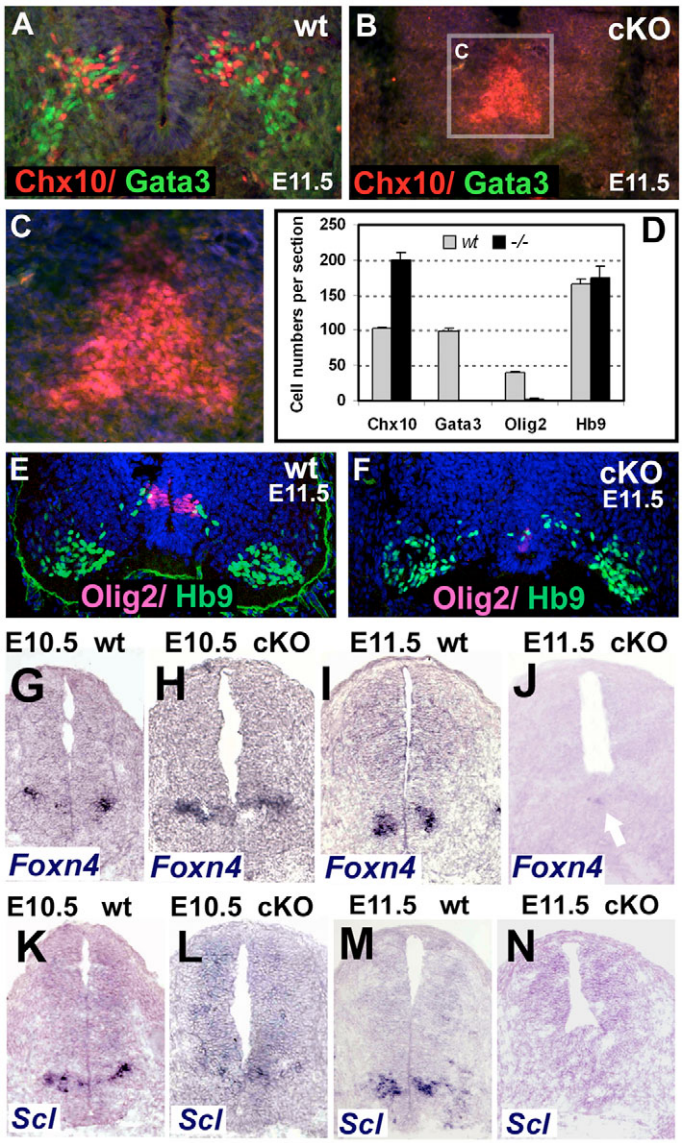

Fig. 7. Notch1 is required for specification of V2b interneurons. Mice carrying a floxed allele of *Notch1* and a *Nestin-Cre* transgene (*Notch1* cKO mice) were analysed at E10.5 and E11.5 by ISH and double immunolabelling for V2a and V2b IN markers. (**A-D**) There is a two-fold increase in the number of Chx10 immunopositive V2a INs in the *Notch1* cKO compared with wild type, whereas Gata3 immunopositive V2b INs are abolished. In addition, the Chx10-positive V2a INs accumulate near the midline of the spinal cord instead of migrating into the parenchyma. (**E**,**F**) Double immunolabelling for Olig2 (magenta) and Hb9 (green). In the *Notch1* cKO, Olig2-positive cells are missing and the Hb9 population is similar to that in the control. Therefore, Notch1 activity is needed for V2b IN production; in the absence of Notch1, V2b INs are respecified as V2a INs with little or no influence on MN fate. (**G-J**) In the *Notch1* cKO, expression of *Foxn4* is increased at E10.5 relative to wild type (compare G with H), but is almost extinguished by E11.5 (I,J). (**K-N**) *Scl* (V2b INs) is reduced at E10.5 (K,L) and absent at E11.5 (M,N) in the *Notch1* cKO. Note that the ventral half of the central canal (and the VZ) is lost in the *Notch1* cKO mouse between E10.5 and E11.5.

so, lose expression of *Foxn4*. Likewise, *Scl* and *Dll4* transcripts were strongly downregulated compared with wild type at E11.5, consistent with their demonstrated dependence on Foxn4 (Fig. 7M,N and data not shown). Cre recombination is thought to be activated at or shortly before E10.5 in the *Nestin-Cre* line (Yang et al., 2006). In keeping with this, the morphology of the spinal cord was normal in the *Nestin-Cre/Notch1*^{flox} at E10.5 (i.e. the ventral VZ was still present). *Foxn4* and *Dll4* were expressed at higher than normal levels in the mutant at E10.5, consistent with the idea that V2a/V2b progenitors are formed prematurely but have not yet had time to differentiate (Fig. 7G,H and data not shown). By contrast, and consistent with the above reasoning, *Scl* was expressed at a reduced level at E10.5 (Fig. 7K,L).

DISCUSSION

Foxn4 activates V2b interneuron development

We found that Foxn4 is both necessary and sufficient to activate *Gata2*, *Scl* and *Gata3*, suggesting that it is near or at the top of the genetic hierarchy that specifies V2b INs. This differs from our previous study, which found that co-electroporation of *Foxn4* together with *Mash1* was necessary to induce ectopic V2b gene expression, *Foxn4* alone being insufficient (Li et al., 2005). At present we are unable to explain this difference but it could perhaps relate to differences in the level of Foxn4 expression achieved following electroporation. We have ruled out functional differences between the *Foxn4* electroporation vectors used because in our hands both constructs give the result reported here. In any case, both our studies demonstrate that Foxn4 is a key determinant of the V2b sub-lineage.

It was shown previously that the transcription factor Scl is necessary and sufficient to induce V2b INs (Muroyama et al., 2005). *Foxn4* transcripts are detected before *Scl* during normal development – at st13 in chick/E9.5 mouse, compared with st16-17 chick/E10.5 mouse (Li et al., 2005; Muroyama et al., 2005) (data not shown), suggesting that *Foxn4* is upstream of *Scl*. Consistent with this, we have now shown that: (1) *Scl* expression is lost in *Foxn4*$^{-/-}$ mice, whereas *Foxn4* expression is unaffected in *Scl*$^{-/-}$ mice; and (2) Foxn4 is able to induce *Scl* expression in chick electroporation experiments. *Foxn4* induces robust expression of *Gata2* in chick neural tube within 24 hours post-electroporation, whereas *Scl* and *Gata3* are not detectable until 48 hours post-electroporation. This temporal order presumably reflects the fact that *Gata2* is required for *Gata3* expression (Karunaratne et al., 2002; Nardelli et al., 1999) and suggests that *Gata2* is genetically upstream of *Scl*. This is backed up by the fact that *Gata2* is expressed ahead of *Scl* during normal development in both chick and mouse (Muroyama et al., 2005) (data not shown). *Gata3* expression is lost in *Scl*-null mice, placing *Scl* upstream of *Gata3* (Muroyama et al., 2005). Taken together, the available data support a genetic cascade *Foxn4* → *Gata2* → *Scl* → *Gata3*. The reduction of *Gata2* expression that was observed in *Scl*-null mice (Muroyama et al., 2005) can be attributed to loss of positive feedback from Gata3 (Karunaratne et al., 2002). A diagram of the proposed network is shown in Fig. 8.

Foxn4 activates Dll4 and Mash1

By loss- and gain-of-function experiments we found that *Foxn4* is necessary and sufficient to activate *Dll4* and *Mash1* expression. We subsequently showed that Mash1 can also induce ectopic expression of *Dll4* in chick spinal cord. This suggests that the conserved Mash1/Brn binding site in the *Dll4* upstream region, reported by Castro et al. (Castro et al., 2006), is functional in vivo and further

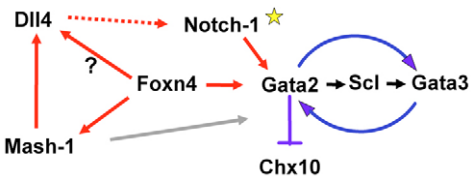

Fig. 8. Genetic interactions in the V2 interneuron lineage. Red arrows represent positive intracellular interactions that we demonstrated in the present study, except for Foxn4 → (?) Dll4, which is speculative. Black arrows denote speculative interactions proposed in the present study. Blue arrows depict interactions demonstrated in previous studies (see below). The dashed red line represents the proposed intercellular Dll4/Notch1 interaction between sibling V2 progenitors, which results in Notch1 being activated (yellow star) in the cells that develop subsequently as V2b INs. The grey arrow signifies the requirement of Mash1 for proper V2b development (Li et al., 2005); this role of Mash1 is ill-defined (see Discussion). Mash1 is sufficient (in chick) but not necessary (in mice) for *Dll4* upregulation (see Discussion). This diagram incorporates observations from a number of studies including the present one: Karunaratne et al. (Karunaratne et al., 2002) demonstrated reciprocal activation of Gata2 and Gata3 and repression of Chx10 by Gata2; Muroyama et al. (Muroyama et al., 2005) showed that Scl induces Gata2 and Gata3 and represses *Chx10* in chick, and that Gata3 is abolished and Gata2 severely reduced in *Scl*-null mice; Li et al. (Li et al., 2005) showed that *Mash1* expression is abolished in *Foxn4*-null mice.

suggested that Foxn4 might activate *Dll4* indirectly through Mash1. However, we found that Mash1 is not required for initiation of *Dll4* expression in the mouse because *Dll4* is expressed normally in the p2 domain of E10.5 *Mash1*-null spinal cord. It is possible that Mash1 might be required to maintain *Dll4* expression after E10.5, but we have not examined older embryos. Alternatively, a requirement for Mash1 in the initiation of *Dll4* expression might be masked in *Mash1* mutant mice through compensatory upregulation of a related proneural factor such as Ngn1 (Neurog1) or Ngn2 (Neurog2). It is also possible that Foxn4 induces *Dll4* directly; in endothelial cells, for example, Foxc1 and/or Foxc2 are known to activate *Dll4* by binding directly to a Fox-binding site in the *Dll4* gene upstream region (Seo et al., 2006).

Apart from regulating *Dll4*, Mash1 must have another role in promoting V2b IN fate, because *Mash1*-null mice at E10.5 are reported to have ~50% less V2b INs than normal (Li et al., 2005), despite the fact that *Foxn4* and *Dll4* are both expressed normally (Fig. 6D and data not shown). More work needs to be done to establish the precise role of Mash1 in V2b IN development.

Notch1 is required for V2b interneuron development

The connection between Foxn4, Dll4 and Mash1 led us to explore the role of Notch-Delta signalling more directly. We previously reported that when Notch1 function was disrupted in the ventral spinal cord, the result was a ~30% overproduction of (Chx10, Lhx3) double-positive V2a INs and an ~18% loss of [Islet 1 (Isl1), Lhx3] double-positive MNs, although the total number of Islet-positive MNs was unchanged (Yang et al., 2006). This was originally interpreted as a fate switch from MN to V2 IN production. However, in the present study we found that Gata3-positive V2b INs were completely lost, whereas the number of Hb9-positive MNs was not changed significantly in the *Notch1* mutant. Therefore, we conclude that the increase in V2a INs is more likely to result from respecification of V2b INs than from respecification of MNs to V2a INs. Since the V2 phenotype of the conditional *Notch1* mutant is analogous to that of the *Foxn4*-null mouse, it appears that both Notch1 and Foxn4 activities are required for V2b IN production.

The default behaviour of p2 progenitors in the absence of Notch1 or Foxn4 activity is to differentiate as V2a INs, suggesting that active Notch1 acts cell-autonomously in collaboration with Foxn4 to drive V2b development. We have not been able to address directly the question of whether Notch1 acts in a cell-autonomous fashion in V2b INs. However, we observed that electroporated *Dll4* is co-expressed with endogenous Chx10 in some V2a INs (Fig. 5C), suggesting that Dll4-mediated inhibition of V2a INs is non-cell-autonomous, as expected from the classical view of Notch-Delta neighbour-to-neighbour signalling. This contrasts with *Foxn4*, which was never co-expressed with Chx10, in keeping with its expected cell-autonomous role. A cell-autonomous role for Notch is indicated by the requirement for presenilin 1 (Psen1) for V2b lineage development (Peng et al., 2007). Psen1 is involved in the intracellular cleavage of Notch (Wines-Samuelson and Shen, 2005). It also fits with the report that Notch1 binds to an enhancer in the upstream region of the *Gata2* gene during hematopoiesis (Robert-Moreno et al., 2005).

Why does Dll4 electroporation inhibit V2a IN production without causing a compensatory increase in V2b INs in p2? Perhaps *dll4* electroporation reduces the total number of V2 INs (V2a +V2b) by inhibiting production of V2 progenitors from their neuroepithelial precursors, while simultaneously biasing the fate of the remaining V2 progenitors from V2a towards V2b. If so, the fact that there is no significant change in the number of V2b INs in our electroporation experiments at st14-16 might be the result of two equal but opposing effects. If this explanation is correct, then the precise outcome of the experiment might depend critically on the time of electroporation, because this could alter the magnitude of one effect versus the other. Consistent with this idea, we found a small reduction in the number of V2b INs (as well as a reduction in V2a INs) when we electroporated at st11-12. Peng et al. (Peng et al., 2007) also found a reduction in total V2 INs in their electroporation experiments at st13. This model is necessarily speculative and other explanations are possible.

Notch1/Dll4 signalling breaks symmetry and splits the V2 lineage

What is the mode of action of Notch1 in V2 IN development? One possibility might be that p2 progenitors normally generate V2a INs first, before switching to V2b production, and that Dll4/Notch1 is needed to keep some progenitors in cycle long enough to generate V2b INs. In that case, eliminating Notch signalling might be expected to cause accelerated differentiation along the V2a pathway and loss of V2b differentiation, as observed. However, there is no evidence that V2a INs are formed before V2b INs. *Chx10* and *Gata3* are both expressed together for the first time at E10.5 in mouse (Liu et al., 1994; Nardelli et al., 1999). V2a and V2b subpopulations are formed simultaneously in chicken too (Karunaratne et al., 2002). We therefore propose that Notch1-Delta signalling has two consecutive or parallel functions in the p2 progenitor domain: (1) it inhibits neuroepithelial (radial) precursors from differentiating prematurely into V2 progenitors; and (2) it segregates V2 progenitors into V2a and V2b sub-lineages, inhibiting V2a and promoting V2b development.

The majority of cells that co-express *Foxn4* and *Dll4* are closely apposed pairs of cells at the ventricular surface, and these are likely to represent the products of recent progenitor cell divisions (Fig. 4A-

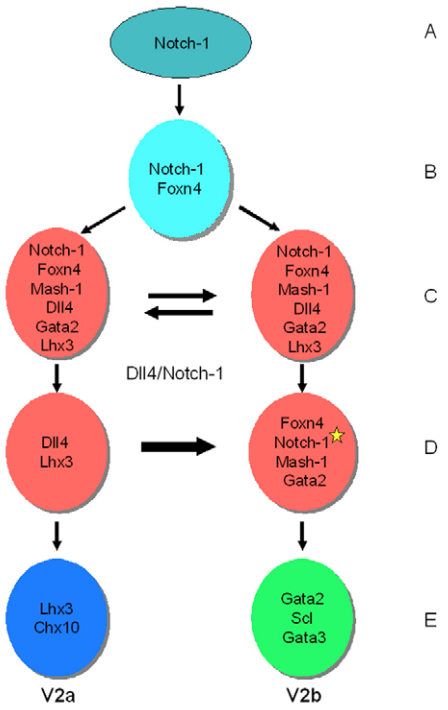

Fig. 9. Generation of V2a and V2b INs from common progenitors in the p2 domain. Multipotent neuroepithelial (radial) progenitors (**A**), which do not express Foxn4, generate a population of V2a/V2b (p2) progenitors (**B**). All V2a/V2b progenitors express Foxn4, which induces the expression of Dll4, Gata2 and Mash1. These common progenitors also start to express Lhx3 at their final division (**C**). Notch1 is expressed in all p2 progenitors (Lindsell et al., 1996), so Notch1/Dll4 reciprocal cell-cell interactions are initiated (opposing arrows in C). This situation resolves into two populations of progenitors, one with activated Notch1 (Notch1*) and the other with Dll4 (**D**). Notch1* blocks the V2a fate and, in cooperation with Foxn4 and Mash1, specifies V2b IN fate (**E**). The complementary set of p2 progenitors (Dll4-positive) that fails to activate Notch1 adopts the V2a fate instead, possibly under the control of Lhx3 (Tanabe et al., 1998) (E). In this way, V2a and V2b INs are generated in a salt-and-pepper fashion during the same time window from a homogeneous population of p2 progenitors.

D). This observation suggests that Dll4/Notch1 interactions involve sibling pairs of cells that have not yet separated after division, and is consistent with the idea that a single V2a/V2b progenitor cell might generate one V2a and one V2b neuron, as illustrated schematically in Fig. 9. Alternatively, bipotential V2a/V2b progenitors might divide asymmetrically to generate a dedicated V2a progenitor and a dedicated V2b progenitor, which can undergo a further symmetrical division(s) before terminal differentiation. Either of these scenarios would be consistent with our observations that approximately equal numbers of V2a and V2b INs are formed under normal circumstances and that twice the normal number of V2a INs form in the absence of Notch1 (Fig. 7D).

Note that our proposed roles for Mash1 and Dll4/Notch1 signalling in separating V2a and V2b lineages is closely analogous to the roles proposed for Mash1 and Dll1/Notch in specifying excitatory and inhibitory (dIL_A and dIL_B) INs in the dorsal spinal cord (Mizuguchi et al., 2006). It is possible that V2a and V2b INs are also a complementary excitatory/inhibitory pair – V2a INs are known to be glutamatergic and excitatory in zebrafish (Kimura et al., 2006), but the neurotransmitter phenotype of V2b INs has not yet been established. It is possible that Notch-Delta signalling might be a general mechanism for creating complementary pairs of INs.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/19/3427/DC1

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