A regulatory network involving Foxn4, Mash-1 and Delta-like 4/Notch-1 generates V2a and V2b spinal interneurons from a common progenitor pool

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Key words: Notch-1, Delta-like 4, Foxn4, Gata, Scl, Chx10, spinal cord, neurogenesis, chick, mouse, V2 interneurons

Running title: Foxn4, Dll4/Notch-1 and Mash-1 specify V2a/V2b spinal interneurons

SUMMARY (188 words)

In the developing central nervous system, cellular diversity depends in part on organizing signals that establish regionally-restricted progenitor domains, each of which produces distinct types of differentiated neurons. However, the mechanisms of neuronal sub-type specification within each progenitor domain remain poorly understood. The p2 progenitor domain in the ventral spinal cord gives rise to two interneuron subtypes called 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 interneurons and is required directly for V2b but not for V2a development. We show here that Foxn4 induces expression of Delta-like 4 (Dll4) and Mash-1/Ascl1. Dll4 then signals through Notch-1 to subdivide the p2 progenitor pool. Foxn4, Mash-1/Ascl1 and activated Notch-1 trigger the genetic cascade leading to V2b interneurons while the complementary set of progenitors, without active Notch-1, generates V2a interneurons. Thus Foxn4 plays a dual role in V2 IN development: 1) by initiating Delta-Notch 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.

INTRODUCTION

The neurons and glial cells of the mature central nervous system (CNS) develop from the neuroepithelial cells that surround the lumen of the embryonic spinal cord and the ventricles of the brain - the so-called ventricular germinal 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 organizing centres (Briscoe et al., 2000; Ericson et al., 1997). For example, graded Sonic hedgehog subdivides the ventral VZ into five progenitor domains known as p3, pMN, p2, p1 and p0 (ventral to dorsal). This initial patterning phase is followed by a neurogenic phase during which the progenitor domains give rise to particular combinations of differentiated neurons and glia (Rowitch, 2004). The interneurons of the ventral cord assemble into local networks that generate the rhythmic output required for locomotion. Their interconnections and synaptic properties are determined during development, by the gene regulatory networks that operate during VZ patterning and neurogenesis (reviewed by Kiehn, 2006). Therefore, to understand how locomotor circuits arise it is necessary to understand the genetic and cellular mechanisms that determine interneuron diversity.

The p2 progenitor domain generates two distinct subtypes of interneurons, so-called V2a and V2b interneurons (INs) (Karunaratne et al., 2002; Li et al., 2005; Smith et al., 2002; Zhou et al., 2000). Post-mitotic V2a INs are characterized by expression of the homeodomain transcription factor

Chx10 (Ericson et al., 1997), whereas V2b INs express transcription factors Gata2, Gata3 and Scl (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 known to be excitatory (glutamatergic) and to project ipsilaterally (Kiehn, 2004; 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 and this paper). How does this homogeneous progenitor pool generate two distinct neuronal sub-types?

The Notch-Delta signalling pathway is often used to establish or to maintain differences between lineally related cells (Artavanis-Tsakonas et al., 1999; Louvi et al., 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. Notch1-3 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 (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 interneuron development (Benedito and Duarte, 2005).

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. We also found that Foxn4 controls *Dll4* and *Mash-1/Ascl-1* expression in p2. In gain of function assays, Dll4 inhibited the development of V2a INs and conversely, when Notch-1 was conditionally inactivated in Nestin-expressing progenitor cells and their derivatives, V2a INs were over-produced at the expense of V2b INs. Taken together, our data suggest the following model: 1) Foxn4 activates *Dll4* and *Mash-1/Ascl1* in common V2a/V2b progenitors; 2) subsequent neighbour-to-neighbour signalling via Dll4 activates Notch-1 in a subset of p2 progenitors, which then generate V2b INs under the combined action of Notch-1, Foxn4 and Mash-1; 3) the complementary set of progenitors fails to activate Notch-1 and consequently generates V2a INs.

RESULTS

Foxn4 is necessary and sufficient for V2b lineage specification, and suppresses V2a 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 interneurons. We analyzed

Foxn4 expression in chick embryos by in situ hybridization (ISH) during Hamburger-Hamilton stages 10 to 25 (st10-st25). We first detected small numbers of *Foxn4*-positive cells in the rostral spinal cord at st13 (Fig. 1A). At later stages the number of *Foxn4* –positive cells increased. As in the mouse, a few cells were present in the ventricular zone (VZ) close to the lumen but most accumulated towards the outer margin of the VZ (Fig. 1B, C). 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 (Fig. 1D and 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* (Fig. 1E, G) but no overlap between *Foxn4* and *Chx10* (Fig. 1F, H), implicating Foxn4 in the development of V2b but not V2a INs.

We tested the ability of Foxn4 to activate expression of V2 specific genes in the chick neural tube. A plasmid vector that expresses full-length mouse Foxn4 under the transcriptional control of the *β-actin* promoter, with a downstream *IRES-GFP* reporter (*β-actin-Foxn4-IRES-GFP*) was electroporated unilaterally in the st12-14 chick spinal cord (Fig. 2). Ectopic expression of *Foxn4* mRNA was confirmed by ISH or by immunohistochemistry for GFP, which was expressed from the same mRNA via an IRES. We found that V2b markers were induced ectopically in all parts of the spinal cord that expressed *Foxn4*. *Gata2* transcription was induced robustly at 24 hours post-electroporation (50/50 embryos after 24 hours and 20/20 after 48 hours) (Fig. 2A, E', F). *Scl* and *Gata3* were detected later, at 48 hours (0/5 embryos after 24 hours versus17/17 embryos after 48 hours for *Scl;* 0/5 embryos after 24 hours versus 6/8 embryos after 48 hours for *Gata3*) (Fig. 2J, 3B, C for *Scl* and Fig. 2D, I for *Gata3*). Foxn4 was unable to induce V2a INs, judging by its failure to induce ectopic expression of Chx10 protein or mRNA (0/15 embryos) or *Lhx3* mRNA (0/7 embryos) after 24 or 48 hours (Fig. 2B, C, E'', G, H). In contrast, the number of Chx10-expressing cells produced from the p2 domain was reduced by 62 ± 9% (mean ± standard error, n=8) on the electroporated side compared to the control side (107 cells on the control side versus 33 on the electroporated side, 41 sections from eight embryos). It is noteworthy that the Chx10-positive V2a INs that were spared in these electroporation experiments did not co-express Foxn4 (Fig. 2G, H), consistent with the idea that Foxn4 acts cell-autonomously to repress the V2a program.

It has been reported that Scl function is necessary and sufficient for V2b interneuron development and is required for the maintenance of normal levels of *Gata2* (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. 3A). *Scl* mRNA expression was abolished in *Foxn4* mutant mouse spinal cords at E10.5 (3/3 embryos) and E11.5 (2/2 embryos)

(Fig. 3D, E and not shown). Conversely, *Foxn4* was expressed as normal in *Scl* conditional null mice (2/2 embryos) (Fig. 3F, G). Also, as described above, *Foxn4* induces *Scl* expression after 48h (17/17 embryos) (Fig.3C). Therefore, it seems that *Foxn4* lies upstream of *Scl* in the genetic hierarchy leading to V2b INs.

In control experiments we electroporated a vector identical to *β-actin-Foxn4*, except that the *Foxn4* coding sequence between the poly-linkers was inverted. In none of the eight embryos analyzed did we find ectopic expression of *Gata2, Gata3* or *Scl* (not shown). Taken together, our data suggest that *Foxn4* might be a master regulator of the V2b lineage. Further, we have shown that Scl lies downstream of Foxn4 in the pathway that governs development of V2b INs.

Ectopic Foxn4 represses interneuron fates outside the p2 domain

Foxn4 induces ectopic expression of V2b markers in the dorsal spinal cord (Fig. 2), raising the possibility that it might be a master regulator of the V2b sub-lineage. To test this further we asked whether Foxn4 can repress alternative fates in the dorsal cord. We electroporated *β-actin-Foxn4-IRES-GFP* into the embryonic chick neural tube at st14 and immunolabelled sections 24 hours later with anti-Engailed1 (En1), which labels postmitotic V1 interneurons (Ericson et al., 1997) and anti-Lhx1/2, which labels postmitotic interneurons derived from progenitor domains dP1-dP6 with the exception of dP3 domain (reviewed by Lewis et al., 2006). Foxn4 was able to repress both of these markers (Fig. 4). A reduction of 31% ± 3% (mean ± standard error, n=4) was observed for En1 (1,173 cells on the control side versus 776 on the electroporated side, 39 sections from four embryos) (Fig. 4A) and a reduction of 45% ± 13% (n=4) for Lhx1/2 (4,239 cells on the control side versus 2,604 on the electroporated side, 30 sections from four embryos) (Fig. 4B). The negative control vector with inverted *Foxn4* sequences had no activity (not shown). Together with the results described in the previous paragraph, this suggests that ectopic expression of Foxn4 reprograms progenitors to a V2b IN fate.

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 in *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-galactosidase protein was present in cells that co-express Chx10 (Fig. 5A) as well as in cells that express Gata3 (Fig. 5B). In contrast, *Foxn4* transcripts or protein were never found in the same cells as Chx10 or Gata3 (Fig. 1H) (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-galactosidase 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. 5D) as well as *Gata2* (Fig. 1G) and *Mash-1* (Fig. 5C, 8A).

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. 6A, B and not shown). Some of the *Dll4*-positive cells in the p2 domain co-express *Foxn4* (Fig. 6A, B). Many of these *Foxn4/ Dll4* double-positive cells are found at the ventricular surface, where mitosis occurs. Double-positive cells frequently occur as cell pairs (arrows in Fig. 6B, shown at higher magnification in C, D). These images suggest strongly that *Dll4* and *Foxn4* are co-expressed in cells that are dividing, or 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 analyzed (Fig. 6F). A control vector with inverted *Foxn4* sequences had no such effect (not shown). Consistent with these observations, *Dll4* expression was abolished in the p2 domain of *Foxn4* null mice at E10.5 (3/3 embryos analyzed) and E11.5 (2/2 embryos analyzed) (Fig. 6E and not shown). We conclude that Foxn4 is necessary and sufficient for activation of *Dll4* in p2 progenitors.

Delta-like-4 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* (*hDll4*) under control of the CMV promoter (*CMV-hDll4-Myc*). We performed two sets of experiments. In the first we electroporated at st11-13 and analyzed the embryos after a further 44h (st19-20). In 16 embryos analyzed, we found no ectopic induction of Chx10 immunoreactivity or *Gata2* mRNA. In 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 to 90 *Gata2*-positive cells on the control side versus 58 on the electroporated side, 24 sections from 4 embryos)(data not shown). In the second set of experiments we electroporated at st14-16 and analyzed the embryos after a further 48h (st21-23). In this set of experiments 15 embryos were analyzed for Chx10 inmunoreactivity and *Chx10*, *Scl* and *Gata2* mRNA (Fig. 7). As in the first experiment, 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 ± standard error. 137 sections from 13 embryos, two-tail t-test=3.6 at p=0.001) (Fig. 7A, B). In these experiments *Gata2* mRNA was expressed ectopically in some embryos (19/63 sections in 5 out of 15 embryos). In general, the induction of *Gata2* was modest and always restricted to the p1-p0 domain (Fig. 7D', white arrow). Despite this small amount of ectopic expression the total amount of *Gata2* signal (estimated by counting pixels with the Image-J program) 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. 7D',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. 7D'', E). These results suggest that at st14-16 Dll4 over-expression specifically represses the V2a fate with little or no effect on V2b fate.

It is perhaps significant that in *Dll4* electroporations some cells were Dll4-Myc/ Chx10 double-positive (Fig. 7C), indicating that expression of Dll4 is compatible with expression of Chx10 in the same cell. This is consistent with the notion that the inhibition of Chx10-positive V2a INs that we observe is through the action of Dll4 on neighbouring cells (i.e. a non cell-autonomous activity of Dll4) - as expected for conventional Delta-Notch signalling.

Foxn4 induces Mash-1/Ascl1 in the p2 domain

The extensive overlap of *Mash-1* and *Foxn4* expression in the mouse p2 domain (Figs. 5C, 8A) suggested some form of regulatory relationship. We therefore explored the interactions between *Foxn4* and *Mash-1* in more detail. We confirmed the finding of Li et al. (2005) that *Foxn4* is expressed as normal in *Mash-1* null spinal cord (Fig. 8C, D). After electroporating *β-actin-Foxn4-IRES-GFP* in the chick spinal cord at st13-14, we found strong ectopic induction of *Cash1* (chick homologue of *Mash-1/Ascl1*) after 24 hours (6/6 embryos) and 48 hours (3/5 embryos; Fig. 8B and not shown). The negative control vector with inverted *Foxn4* sequences had no activity (not shown). These experiments indicate that *Foxn4* is upstream of and controls expression of *Mash-1* in p2, and fits with the observation that *Mash-1* expression in p2 is lost in *Foxn4* null mice (Li et al.2005).

Mash-1/Ascl stimulates Dll4 expression but does not induce V2b INs

It has been shown that Mash-1 controls the expression of *Dll1* in the ventral telencephalon and dorsal spinal cord (Casarosa et al., 1999), so we asked whether Mash-1 can also induce *Dll4*. We electroporated full-length mouse *Mash-1* under the transcriptional control of a synthetic *β-actin* promoter (*CAGGS-Mash1-IRES-GFP)* into st13-14 chick neural tube and confirmed expression of

Mash-1 by ISH and immunohistochemistry for GFP (Fig. 8B, E, F, H and not shown). After 24 hours of incubation 8/8 embryos showed clear ectopic induction of *Dll4* on the electroporated side (Fig. 8E). After 48 hours 5/5 embryos displayed weaker but still clear induction of *Dll4* (not shown). In none of the thirteen embryos analyzed did we find any ectopic expression of *Chx10, Gata2* or *Scl* transcripts or Chx10 immunoreactivity (not shown, Fig. 8H', H'', F, G respectively). On the other hand, we found a loss of endogenous Chx10-positive INs in the p2 domain of 5/5 embryos analyzed (76 ± 6% reduction, n = 23) (Fig. 8F, F', G), with little or no concomitant reduction of *Gata2* or *Scl* (Fig. 8H', H''). These data suggested that induction of *Dll4* and consequent repression of Chx10-positive V2a INs by Foxn4 might be mediated indirectly via Mash-1/Ascl1. However, we have found that *Dll4* is expressed as normal at E10.5-11 in *Mash-1* null embryos (4/4 embryos; data not shown). Therefore, Mash-1 might be involved in maintaining or reinforcing *Dll4* expression but is not required for its initiation. Although Mash-1 is absolutely 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.

Notch-1 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 analyzed *Notch-1* mutant (cKO) embryos at E10.5 and E11.5 by ISH for *Foxn4* or *Scl*, or by immunohistochemistry for Chx10, Gata3, Olig2 or Hb9 (Fig. 9). Olig2 is a basic helix-loop-helix transcription factor that is expressed in the progenitors of motor neurons (MNs) and oligodendrocytes but not in post-mitotic MNs (Lu et al., 2000), while Hb9 is a transcription factor expressed in early committed MNs (Thaler et al., 1999). At E11.5 no Gata3 (zero versus 99 ± 3, n=14 sections from 3 embryos, two–tail t-test=26, $p<0.001$) or *Scl*-positive cells were present in the ventral spinal cord of *Notch-1* cKO mice (3/3 embryos analyzed) (Fig. 9A-D, M-N). Instead, twice the normal number of Chx10-positive cells was observed (200 ± 10 versus 102 ± 3, n=16 sections from 3 embryos, two-tail t-test=8.6 at $p<0.001$) (Fig. 9A-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 3 embryos, two-tail t-test= 17, $p<0.001$) but the number of Hb9-positive cells was not significantly affected in the *Notch-1* mutant (3/3 embryos analyzed) with respect to wild type mice (175 ± 16 versus 165 ± 8, n=14 sections from 3 embryos, two-tail t-test=0.6, p=0.6) (Fig. 9D, E-F). It seems that Notch-1 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 Notch-1 does not seem to result in re-specification of pMN progenitors to p2 progenitors, as originally proposed (Yang et al., 2006). Rather, the phenotype is

more consistent with re-specification of V2b to V2a INs, consistent with the idea that signalling through Notch-1 is required for V2b IN development.

Foxn4 is very much reduced in the E11.5 *Notch-1* conditional null spinal cord (Fig. 9I, 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 so, lose expression of *Foxn4*. Likewise, *Scl* and *Dll4* transcripts were strongly down-regulated compared to normal at E11.5, consistent with their demonstrated dependence on Foxn4 (Fig. 9M-N and 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/ Notch-1*^{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. 9G-H and not shown). In contrast, and consistent with the above reasoning, *Scl* was expressed at a reduced level at E10.5 (Fig. 8K-L).

DISCUSSION

Foxn4 activates V2b interneuron development

In this study we found that Foxn4 is both necessary and sufficient to activate *Gata2*, *Scl* and *Gata3,* suggesting that it is 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 *Mash-1* 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 relate, perhaps, to differences in the level of Foxn4 expression achieved following electroporation. We have ruled out functional differences between the *Foxn4* electroporation vectors themselves 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 to st16-17 chick/ E10.5 mouse (Li et al., 2005; Muroyama et al., 2005 and 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 chicks and mice (Muroyama et al., 2005 and 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. 10.

Foxn4 activates Dll4 and Mash-1/Ascl

By loss- and gain-of-function experiments we found that *Foxn4* is necessary and sufficient to activate *Dll4* and *Mash-1* expression. We subsequently showed that Mash-1 also can induce ectopic expression of *Dll4* in chick spinal cord. This suggests that the conserved Mash-1/Brn binding site in the *Dll4* upstream region, recently reported by Castro et al. (2006), is functional in vivo and further suggested that Foxn4 might activate *Dll4* indirectly through Mash-1. However, we found that Mash-1 is not required for initiation of *Dll4* expression in the mouse because *Dll4* is expressed normally in the p2 domain of E10.5 *Mash-1* null spinal cord. It is possible that Mash-1 might be required to maintain *Dll4* expression after E10.5 but we have not examined older embryos. Alternatively, a requirement for Mash-1 in the initiation of *Dll4* expression might be masked in *Mash-1* mutant mice through compensatory up-regulation of a related proneural factor such as Ngn1 or Ngn2. 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*, Mash-1 must have another role in promoting V2b IN fate, because *Mash-1* 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. 8D and not shown), More work needs to be done to establish the precise role of Mash-1 in V2b IN development.

Notch-1 is required for V2b interneuron development

The connection between Foxn4, Dll4 and Mash-1/Ascl1 led us to explore the role of Delta-Notch signalling more directly. We previously reported that when Notch-1 function was disrupted in the ventral spinal cord by expression of a *Nestin-Cre* transgene in floxed *Notch-1* mice, the result was a ~30% overproduction of (Chx10, Lhx3) double-positive V2a INs and an ~18% loss of (Islet1, Lhx3) double-positive MNs (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

Notch-1 mutant. Therefore we conclude that the increase in V2a INs is more likely to result from re-specification of V2b INs than from re-specification of MNs to V2a INs. Since the V2 phenotype of the conditional *Notch-1* mutant is analogous to that of the *Foxn4* null mouse, it appears that both Notch-1 and Foxn4 activities are required for V2b IN production.

Loss of Gata3-expressing V2b INs in the *Notch-1* mutant is as expected if activated Notch-1 is required for V2b development. *Notch-1* mutation does not abolish the onset of *Foxn4* expression, since *Foxn4* is genetically upstream of Notch-Delta. The default behaviour of p2 progenitors in the absence of Notch-1 or Foxn4 activity is to differentiate as V2a INs, suggesting that active Notch-1 acts cell-autonomously in collaboration with Foxn4 to drive V2b development. We have not been able to address directly the question of whether Notch-1 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. 7C), suggesting that Dll4-mediated inhibition of V2a INs is not cell-autonomous - as expected from the classical view of Delta-Notch neighbour-to-neighbour signalling. It 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 (PS1) for V2b lineage development (Peng et al., 2007). PS1 is involved in the intracellular cleavage of Notch (Wines-Samuelson and Shen, 2005).

Interpretation of the phenotype of the *Notch-1* mutant is complicated by the fact that the ventral VZ is obliterated between E10.5 and E11.5 (Fig. 9). This presumably reflects loss of neuroepithelial precursor cells in the ventral cord by premature differentiation into post-mitotic neurons, including MNs and V2a INs (Yang et al., 2006 and this paper).

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. (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.

Dll4/Notch-1 signalling breaks symmetry and splits the V2 lineage

We followed the fates of *Foxn4*-expressing progenitors directly in heterozygous *Foxn4 (+/-)* mice, which express *LacZ* under *Foxn4* transcriptional control. The encoded β-gal protein was found in both V2a and V2b INs, demonstrating that both V2 subtypes descend from *Foxn4*-positive progenitors. In addition, *Foxn4* is co-expressed with markers of both V2a and V2b INs (*Gata2, Lhx3, Mash-1*) at the ventricular surface, where progenitor cell mitosis occurs. It therefore seems probable that there is a population of bipotential, Foxn4-positive V2 progenitors that generates V2a and V2b INs simultaneously under the action of Notch-Delta.

What is the mode of action of Notch-1 in V2 interneuron development? One possibility might be that p2 progenitors normally generate V2a INs first, before switching to V2b production, and that Dll4/Notch-1 is needed to keep some progenitors in cycle long enough to generate V2b INs. In that case, abrogation of 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). Karunaratne et al. (2002) found that V2a and V2b subpopulations are formed simultaneously in chicken too.

We therefore propose that Delta/Notch-1 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. It is interesting that the Notch-1 intracellular domain has been shown to bind directly to an enhancer element in the *Gata2* gene promoter during haematopoietic development (Robert-Moreno et al., 2005). This might explain the ectopic Gata2 induction we sometimes observed in *dll4*-electroporated embryos (Fig. 7D') and also suggests that *Gata2* expression might be controlled by different factors sequentially during V2b development - first by Foxn4 in the common V2a/V2b progenitors, then by activated Notch-1 in dedicated V2b precursors and finally by positive feedback from Gata3 in differentiated V2b INs (Karunaratne et al., 2002).

The majority of cells that co-express *Foxn4* and *Dll4* are closely-apposed pairs of cells at the ventricular surface, which likely represent the products of recent progenitor cell divisions (Fig. 6A-D). This observation suggests that Dll4/ Notch-1 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. 11. 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 Notch-1 (Fig. 9D).

Note that our proposed roles for Mash-1 and Dll4/Notch-1 signalling in separating V2a and V2b lineages is closely analogous to the roles proposed for Mash-1 and Dll1/Notch in specifying excitatory and inhibitory (dIL_A and dIL_B) interneurons 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) and mice (Kiehn, 2004) but the neurotransmitter phenotype of V2b INs has not yet been established. Together, our studies and those of Mizuguchi et al. (2006) suggest that Delta-Notch signalling might be a general mechanism for creating complementary pairs of interneurons.

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), *Notch-1* conditional nulls (Yang et al., 2006), *Mash-1 (-/-)* (Guillemot et al., 1993). The *Scl* and *Notch-1* conditional nulls were crossed to *Nestin-Cre* to eliminate the floxed alleles throughout the CNS.

Electroporation constructs

The complete mouse *Foxn4* (*mFoxn4)* open reading frame (ORF) was cloned into the *pCAß-LINK-IRESeGFPm5-ClaI* bi-cistronic expression vector (Schubert and Lumsden, 2005). The *Mash-1* vector was a gift from Francois Guillemot. It contains the coding sequence of mouse Mash-1 under transcriptional control of a synthetic β-actin promoter (*CAGGS*), followed by *IRES-eGFP* (with a nuclear localization signal).

A human *Dll4* (*hDll4*) expression vector was kindly provided by Ji-Liang Li (John Radcliffe Hospital, University of Oxford). The full-length human *Dll4* (hDll4) coding sequence was PCR-amplified from human placental cDNA, using primers 5'-ggatcccatatggcggcagcgtcccgtagcgcct-3' and 5'-accggttcccgcggtacctccgtggcaatgacacattcattc-3'. *hDll4* was released from the pGEM-T Easy vector (Promega) by BamHI/ SacII 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 phosphate-buffered saline (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 ms 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 and Connie Cepko), 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), mouse monoclonal anti-Hb9 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-β-gal at 1:2000 (Cappel, ICN Pharmaceuticals), mouse anti-Lhx2 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 visualize cell nuclei before mounting.

In situ hybridization

Our ISH protocols are as described (http://www.ucl.ac.uk/~ucbzwdr/richardson.htm). The chick *Foxn4* template was cloned by RNA ligase-mediated rapid amplification of 5' and 3' cDNA ends (RLM-RACE) (Gene Racer kit), using the Gene Racer 5' upper primer and 5' ggcagagtgtggagaggaggtgtc 3'. The cDNA product was 850 bp in length. The template for chicken *Dll4* was plasmid *ChEST714c11* (ARK-Genomics). The mouse *Foxn4* probe includes the ORF minus the first 1000 bps, plus the entire 3' UTR sequence. The *LacZ* probe was a 3.7 kb BamHI fragment of the *LacZ* gene (provided by L. Flores Garcia). The mouse *Scl* probe has been described (Muroyama et al., 2005).

ACKNOWLEDGEMENTS

We thank our colleagues at UCL - especially Lisbeth Flores-Garcia and also Huiliang Li, Nicoletta Kessaris and Marcus Fruttiger for helpful discussions and insights, and Kamal Sharma for sharing data prior to publication. We also thank Ji-Liang Li for the human *Dll4* expression vector, Francois Guillemot for the *Mash-1* vector and *Mash-1* mutant mouse embryos, Thomas Jessell and Connie Cepko for anti-Chx10 antibodies, Charles D Stiles for anti-Olig2 and the following for DNA

templates: Luis Puelles for chick *Nkx6.1*, Graham Goodwin for chick *Scl*, Thomas Reh for *Cash-1*, Henrique Domingos for mouse *Dll4*, Janette Nardell for mouse *Chx10* and Stuart Orkin for mouse *Gata2*. Raquel Taveira-Marques is supported by a studentship from the Portuguese Fundação para a Ciência e a Tecnologia. This work was supported by grants from the US National Institutes of Health [R01 NS047572 (DHR) and R01 NS042818 (JS)], the Wellcome Trust and the UK Medical Research Council (WDR).

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FIGURE LEGENDS

Figure 1 Characterization of *Foxn4* expression in the embryonic chick spinal cord. All images are of single or double in situ hybridization signals. The number of *Foxn4*-positive cells in the ventral cord increases between st13 and st22 (A-C). At all stages, the *Foxn4* expressing cells are in the VZ or at the lateral border of the VZ (e.g. panel C). (D) *Nkx6.1* (red)-*Foxn4* (green) at st22. Foxn4-positive cells all fall within the *Nkx6.1* expression domain, which marks the dorsal limit of the p2 progenitor domain. (E) *Gata2* (red)-*Foxn4* (green) at st25. Many cells are double-positive. A higher magnification image of the indicated area in (E) is reproduced in (G), showing that *Gata2* persists longer in the V2b lineage than *Foxn4*, so that there are more *Gata2*-only cells in more lateral positions, further from the lumen. In (G), the boxed cells at the ventricular surface are reproduced, fluorescence channels separate, at the top right of the panel. (F) *Chx10* (red)- *Foxn4* (green) at st22. A higher-magnification image of the indicated area in (F) is reproduced in (H), showing that there is no co-localization between *Chx10* and *Foxn4*.

Figure 2 *Foxn4* is sufficient to induce V2b and suppress V2a interneurons. 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 ISH for *Foxn4* or immunolabelling for GFP (panels marked "Foxn4-GFP"). Consecutive sections are labelled (A, A', A'' etc.). Different fluorescence channels of the same micrograph are labelled (A, A1, A2 etc.). These same conventions are used in all subsequent figures. 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, arrow) 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).

Figure 3 *Foxn4* lies upstream of *Scl* in V2b interneuron development. (A) Double in situ for *Scl* (green)-*Foxn4* (red). Confocal image of wild type E10.5 mouse spinal cord, showing co-localization of *Scl* and *Foxn4* in some cells. (B-C) Sections from chicken embryos electroporated at st12-14 with *β-actin-Foxn4-IRES-GFP* and analyzed after a further 24 (B) or 48 hours (C). *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) *Scl* expression in the p2 domain is dependant on functional Foxn4. Consecutive sections from *Foxn4* null mouse embryos at E10.5 were hybridized in situ 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

visualized by ISH at E11.5 in wild type (F) and *Scl* conditional null mouse spinal cords (G) (see Methods). Taken together, these data demonstrate that *Foxn4* is genetically upstream of *Scl*.

Figure 4 Ectopic Foxn4 expression represses interneuron fates other than V2a. Chick embryos were electroporated at st14 with *β-actin-Foxn4-IRES-GFP* and analyzed after 24 hours by double immunolabelling with anti-GFP (green) and either anti-En1 or anti-Lhx1/2 (red). The numbers of both En1-positive (A) and Lhx1/2-positive (B) cells was reduced (see text for quantification).

Figure 5 *Foxn4* is expressed in common precursors of V2a and V2b INs. (A, B) *Foxn4 (+/-)* embryos were labelled by double immunohistochemistry for β-galactosidase (β-gal, green) and either Chx10 or Gata3 (red) (see text for details). 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 INs. Consistent with this conclusion, *Foxn4*-positive progenitors co-express *Mash-1* (C) and *Lhx3* (D), markers that later segregate into V2b and V2a INs respectively.

Figure 6 *Foxn4* is necessary and sufficient to induce *Dll4* in the p2 domain. (A-C) Double ISH for *Dll4* (green)-*Foxn4* (red) in wild type E10.5 mouse embryos, counter-stained with Hoechst stain to visualize cell nuclei. (A) is a transverse section of spinal cord and (B) a longitudinal section. *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 which 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) Foxn4 induces ectopic expression of *Dll4* in electroporated st11-13 chick neural tube (double ISH for *Foxn4* (green) and *Dll4* (red).

Figure 7 Dll4 inhibits V2a lineage progression. (A and D) Chick embryos were electroporated with human *Dll4-Myc* at st14-16 and analyzed after 48 hours. (A, B) double immunolabelling for Chx10 (red) and Dll4-Myc (green) showing repression of Chx10-positive cells. (C) Some *Dll4*-electroporated cells co-express Chx10, consistent with the idea that Dll4 can suppress V2a generation in a cell-non-autonomous fashion. (D) Immunolabelling for Dll4-Myc (green). (D') Dll4 exceptionally can induce *Gata2.* (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* (E) by pixel-counting software showed no significant effect on V2b production (see text for statistics).

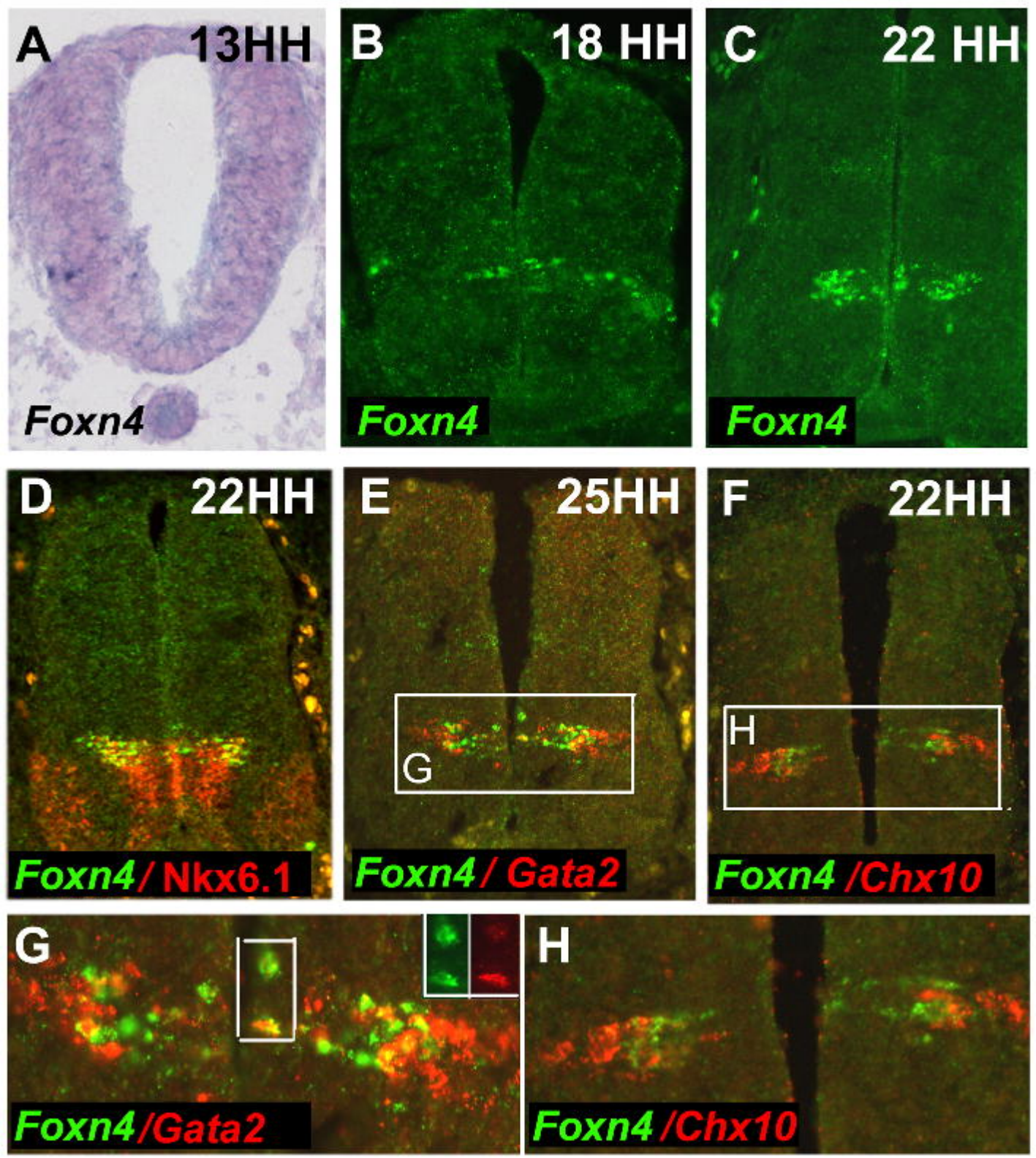
Figure 8 Foxn4 controls *Mash*-1 expression. (A) Double ISH for *Foxn4* (red) and *Mash-1* (green) in E10.5 mouse cord. Note the extensive overlap in the p2 domain. (B, B') Foxn4 induces ectopic expression of *Cash-1* (chick homologue of *Mash-1*) in chick electroporation experiments. (C, D) *Foxn4* expression does not depend on *Mash-1,* because there is no noticeable change in the *Foxn4* ISH signal in *Mash-1* null mice compared to wild type. (E, E') Electroporation of *β-actin-Mash1-IRES-GFP* in the st13-14 chick neural tube induces *Dll4* after 24 hours. Mash-1 expression was confirmed by GFP immunolabelling (E) and *Dll4* by ISH (E'). Mash-1 did not induce ectopic Chx10 but repressed endogenous Chx10 V2a INs in the p2 domain (F', G). Finally, Mash-1 did not induce ectopic V2b markers *Gata2* or *Scl* (H', H'').

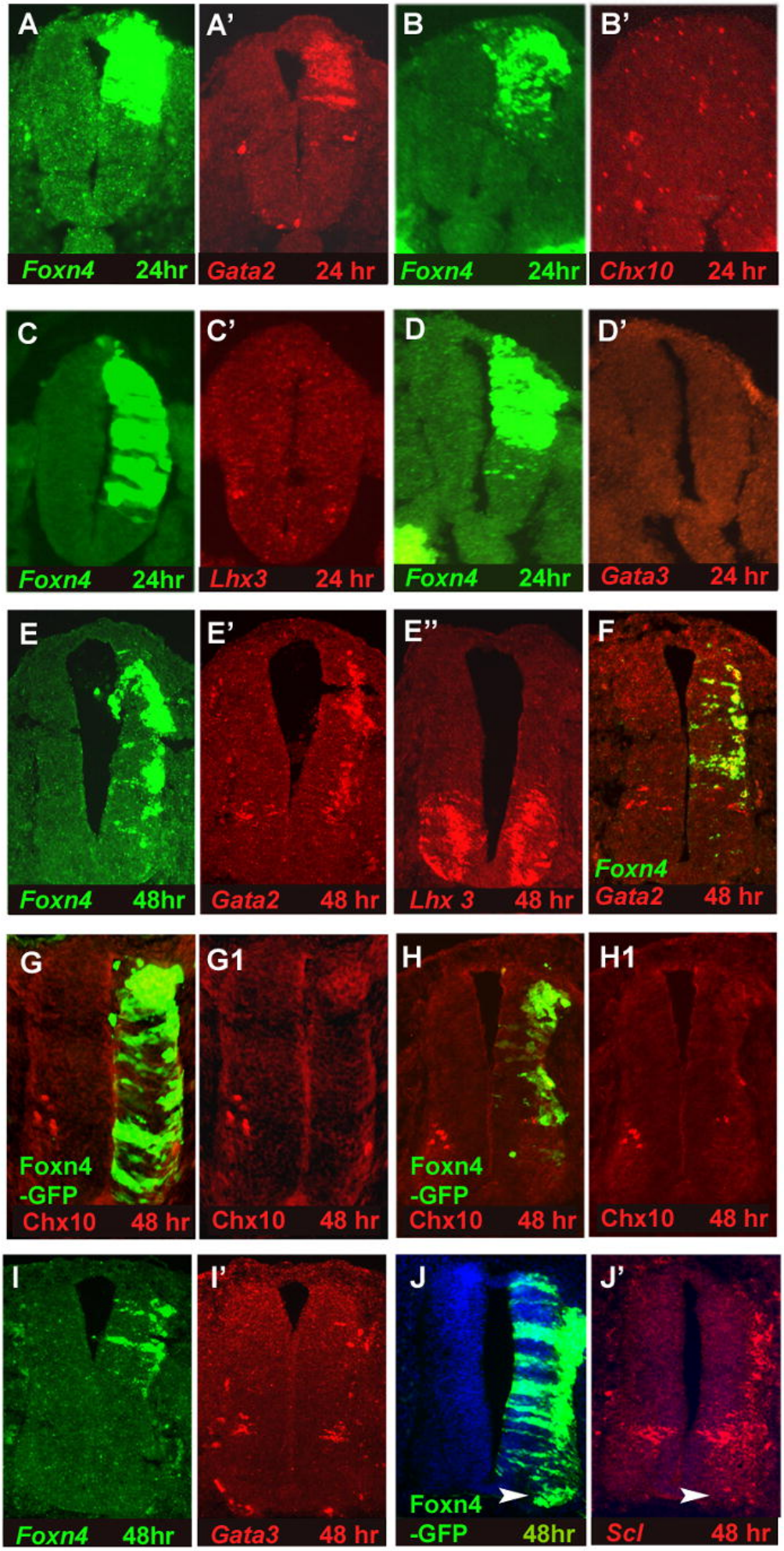
Figure 9 Notch-*1* is required for specification of V2b interneurons. Mice carrying a floxed allele of *Notch-1* and a *Nestin-Cre* transgene (*Notch-1* cKO mice) were analyzed at E10.5 and E11.5 by ISH and double immunolabelling for V2a and V2b interneuron markers. (A-D) There is a two-fold increase in the number of Chx10 immuno-positive V2a INs in the *Notch-1* cKO compared to wild type, while Gata3 immuno-positive 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. (D-F) Double immunolabelling for Olig2 (red) and Hb9 (green). In the *Notch-1* cKO, Olig2-positive cells are missing and the Hb9 population is similar to control. Therefore, Notch-1 activity is needed for V2b IN production; in the absence of Notch-1 V2b INs are re-specified as V2a INs with little or no influence on MN fate. In the *Notch-1* cKO, expression of *Foxn4* is increased at E10.5 relative to wild type (compare G, H) but is almost extinguished by E11.5 (I, J). *Scl* (V2b INs) is reduced at E10.5 (K, L) and absent at E11.5 (M, N). Note that the ventral half of the central canal (and the VZ) is lost in the *Notch-1* cKO mouse between E10.5 and E11.5.

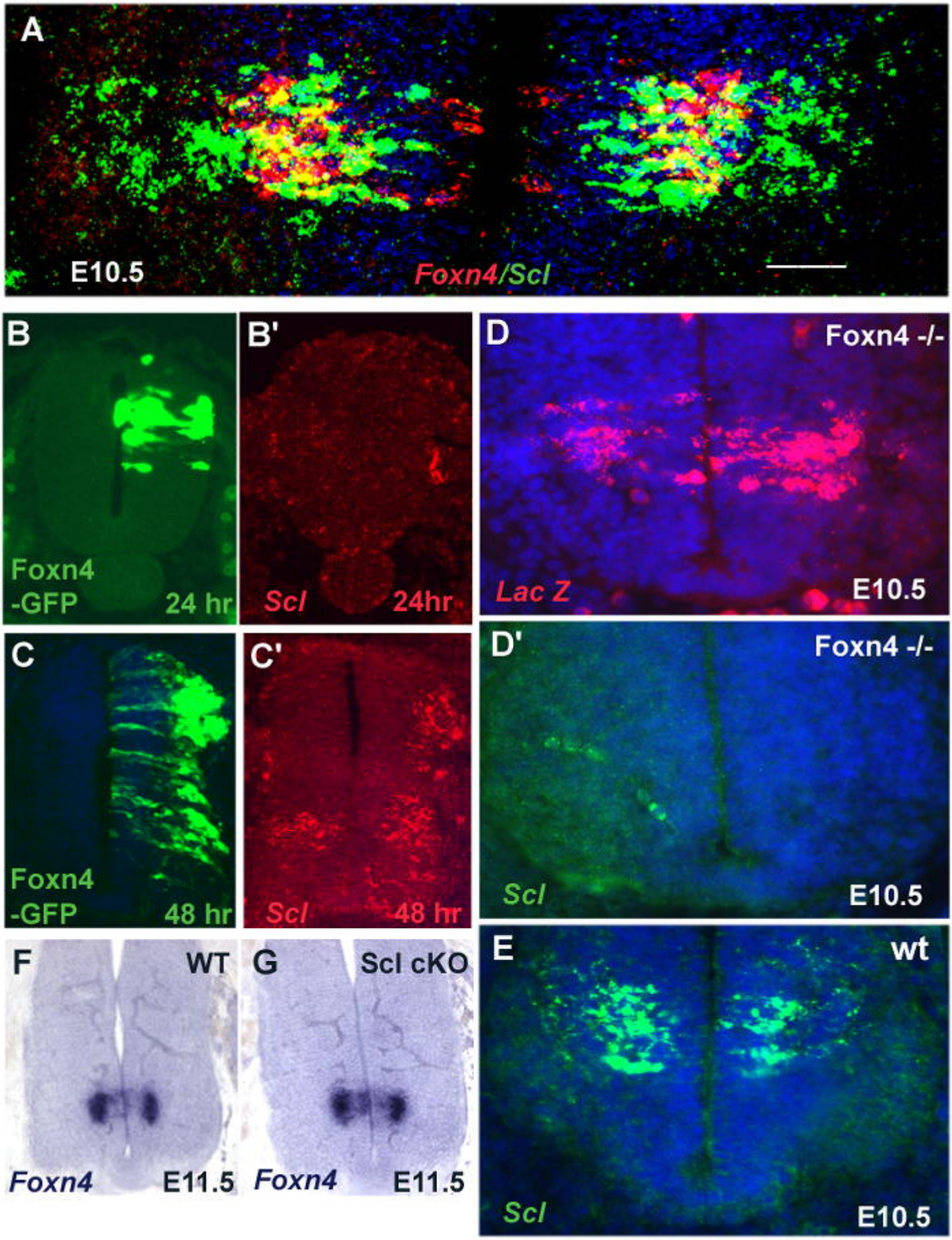
Figure 10 . Genetic interactions in the V2 interneuron lineage. Solid lines represent intracellular interactions in V2a/V2b progenitors and their progeny that cause them to develop along the V2b pathway. Red arrows represent positive intracellular interactions that we demonstrated in the present study. Black arrows represent speculative interactions proposed in the present study. Blue arrows depict intracellular interactions demonstrated in previous studies. The dotted red line represents the proposed Dll4/Notch-1 ligand/receptor interaction between sibling V2 progenitors, which results in Notch-1 being activated (yellow star) in the cells that consequently develop as V2b interneurons. The grey arrow from Mash-1 represents the requirement of Mash-1 for proper V2b development (Li et al., 2005). This role of Mash-1 is ill-defined (see Discussion). Mash-1 is also sufficient but not necessary for *Dll4* up-regulation (see Discussion) This diagram attempts to incorporate observations from a number of studies including the present one: Karunaratne et al. (2002) demonstrated reciprocal activation of Gata2 and Gata3 and repression of Chx10 by Gata2; Muroyama et al. (2005) showed that Scl induces Gata2 and Gata3 and represses *Chx10* in chick,

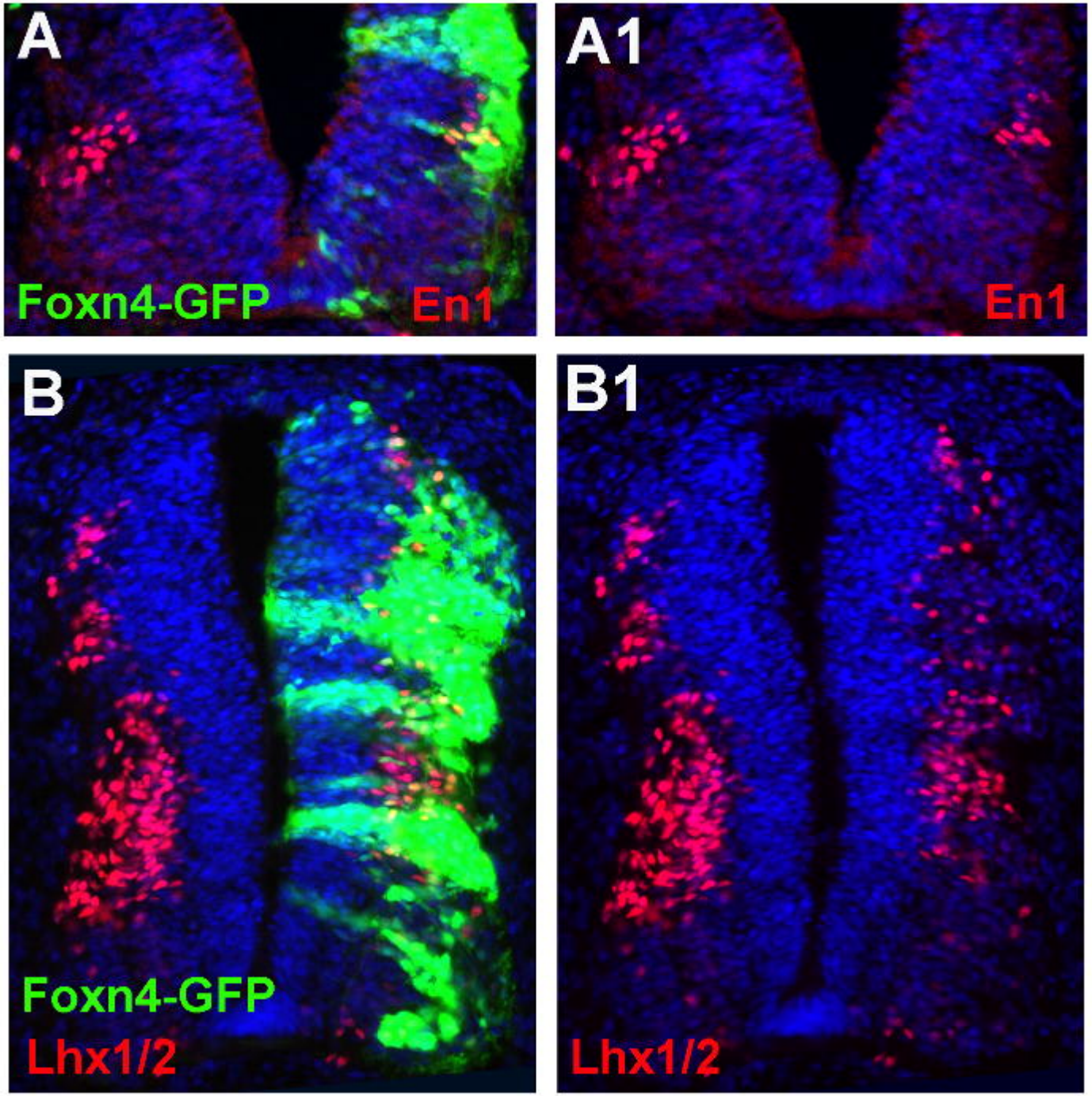
and that Gata3 is abolished and Gata2 severely reduced in *Scl* null mice; Li et al. (2005) showed that *Mash-1* expression is abolished in *Foxn4* null mice.

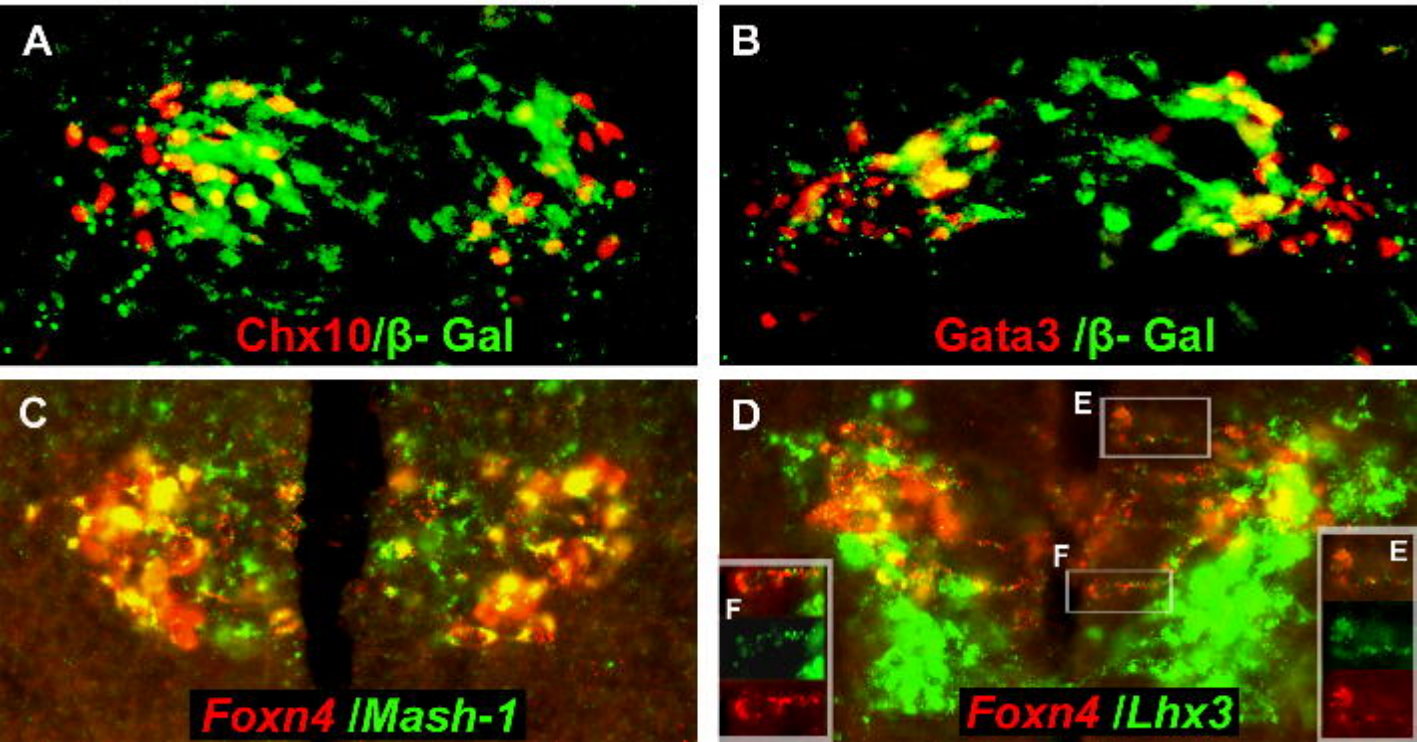
Figure 11 Generation of V2a and V2b INs from common progenitors in the p2 domain. Multipotent neuroepithelial (radial) progenitors (A), which do not express Foxn4, generate a population of V2a/V2b (p2) progenitors (B). All V2a/V2b progenitors express Foxn4, which induces the expression of Dll4, Gata2 and Mash-1. These common progenitors also start to express Lhx3 at their final division (C). Notch-1 is expressed in all p2 progenitors (Lindsell et al., 1996), so Notch-1/ Dll4 reciprocal cell-cell interactions are initiated (opposing arrows in C). This situation resolves into two populations of progenitors, one with activated Notch-1 (Notch-1*) and the other with Dll4 (D). Notch-1* blocks the V2a fate and, in cooperation with Foxn4 and Mash-1, specifies V2b IN fate (E). The complementary set of p2 progenitors (Dll4-positive) that fails to activate Notch-1 adopts the V2a fate instead, possibly under the control of Lhx3 (Tanabe et al., 1998) (E). In this way V2a and V2b interneurons are generated in salt-and-pepper fashion during the same time window from a homogeneous population of p2 progenitors.

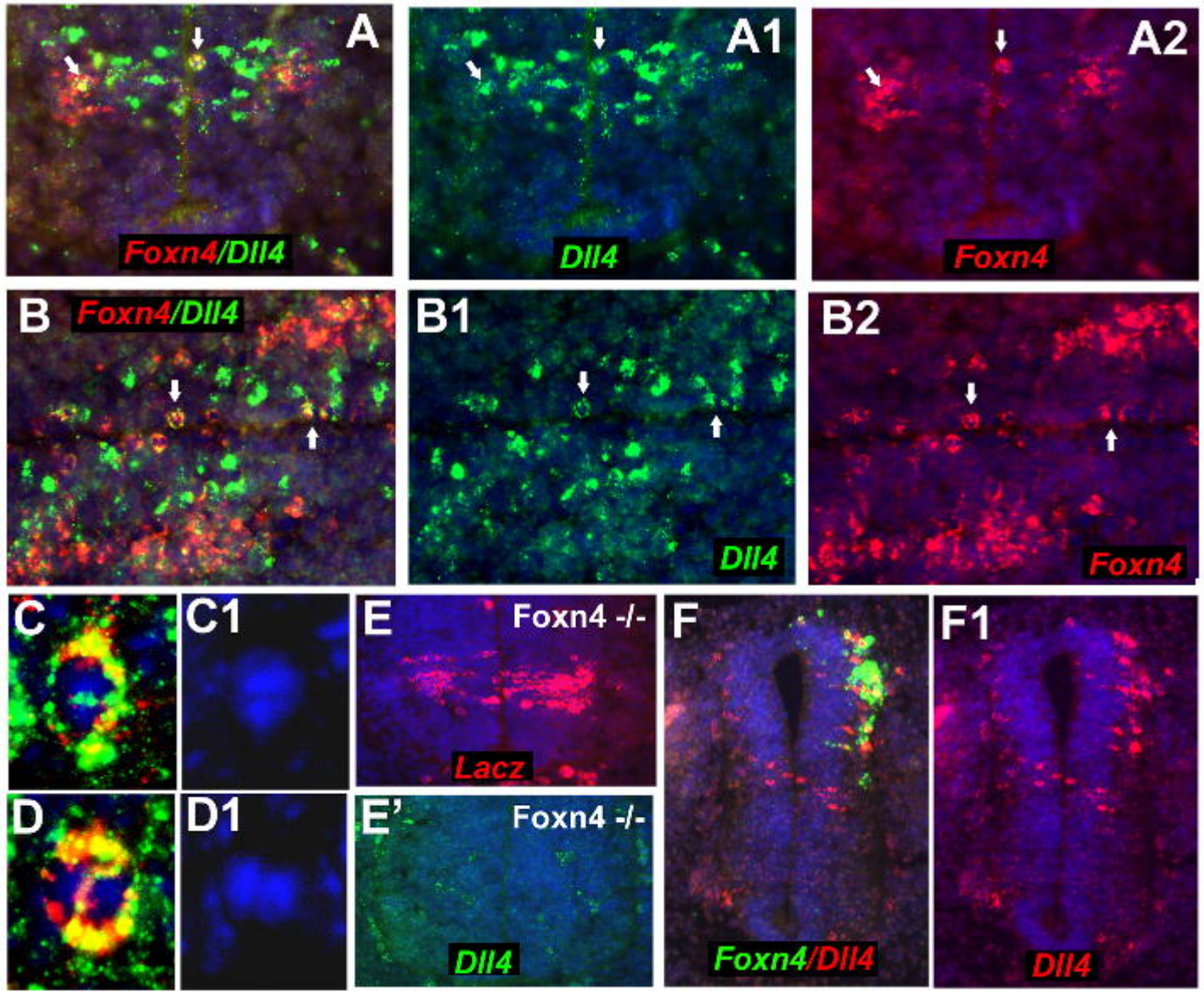

A
13HH
Foxn4
B
18 HH
Foxn4
C
22 HH
Foxn4
D
22HH
Foxn4/ Nkx6.1
E
25HH
G
Foxn4/ Gata2
F
22HH
H
Foxn4 /Chx10
G
Foxn4/Gata2
H
Foxn4 /Chx10

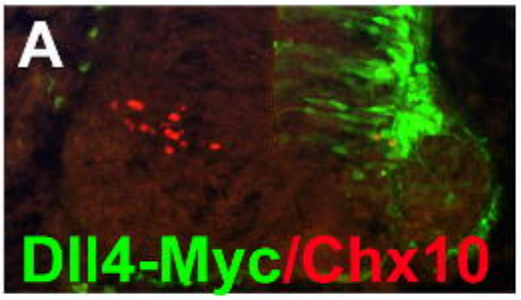

A
A'
B
B'
Foxn4 24hr
Gata2 24 hr
Foxn4 24hr
Chx10 24 hr
C
C'
D
D'
Foxn4 24hr
Lhx3 24 hr
Foxn4 24hr
Gata3 24 hr
E
E'
E''
F
Foxn4 48hr
Gata2 48 hr
Lhx 3 48 hr
Foxn4
Gata2 48 hr
G
G1
H
H1
Foxn4
-GFP
Chx10 48 hr
Chx10 48 hr
Foxn4
-GFP
Chx10 48 hr
Chx10 48 hr
I
I'
J
J'
Foxn4 48hr
Gata3 48 hr
Foxn4
-GFP 48hr
Scl 48 hr

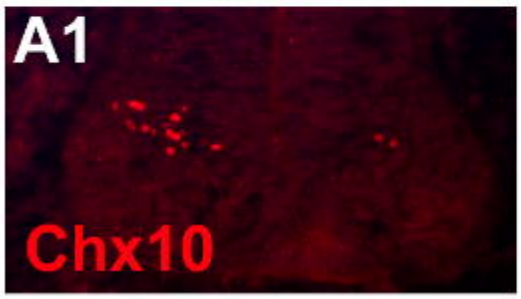

A
E10.5
Foxn4/Scl
B
Foxn4
-GFP
24 hr
B'
Scl
24hr
C
Foxn4
-GFP
48 hr
C'
Scl
48 hr
D
Foxn4 -/-
Lac Z
E10.5
D'
Foxn4 -/-
Scl
E10.5
E
wt
Scl
E10.5
F
WT
Foxn4
E11.5
G
Scl cKO
Foxn4
E11.5

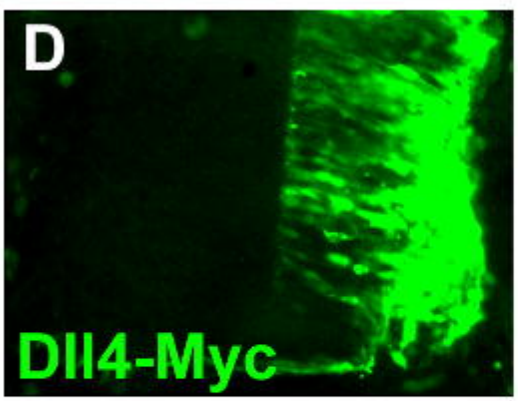

A
Foxn4-GFP
En1
A1
En1
B
Foxn4-GFP
Lhx1/2
B1
Lhx1/2

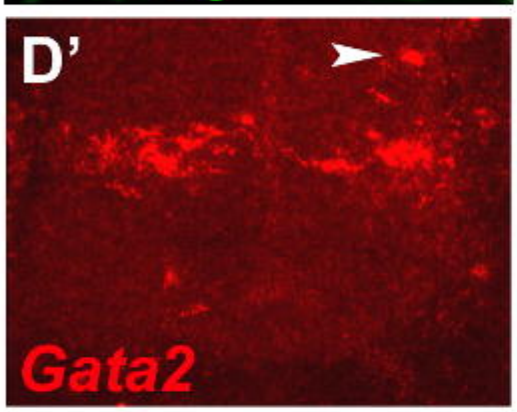

A
Chx10/β- Gal
B
Gata3 /β- Gal
C
Foxn4 /Mash-1
D
E
F
F
E
Foxn4 /Lhx3


A
Foxn4/Dll4
A1
Dll4
A2
Foxn4
B
Foxn4/Dll4
B1
Dll4
B2
Foxn4
C
C1
D
D1
E
Foxn4 -/-
Lacz
E'
Foxn4 -/-
Dll4
F
Foxn4/Dll4
F1
Dll4

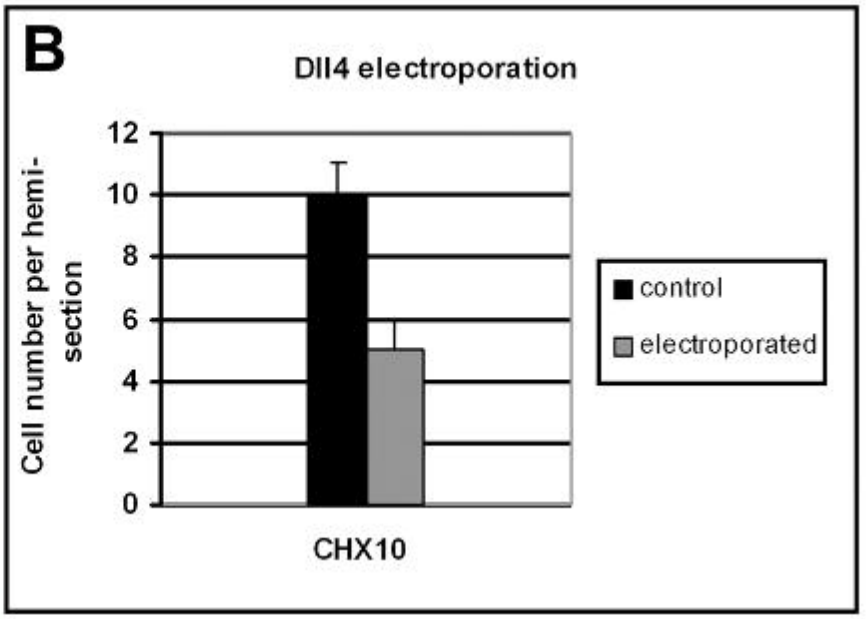

A
Dll4-Myc/Chx10

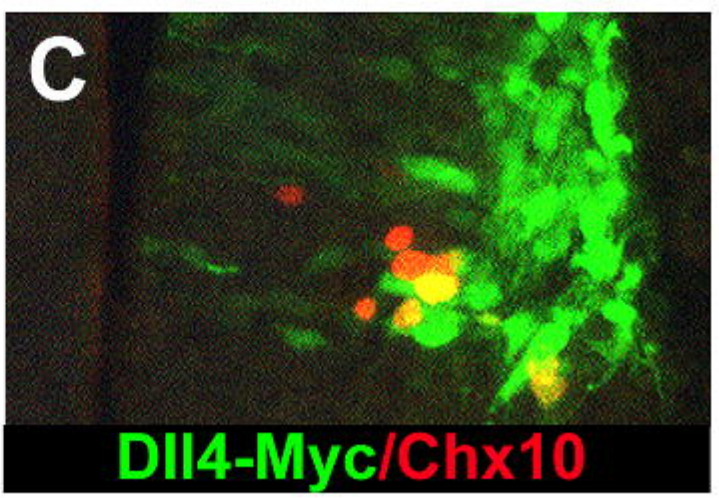

A1
Chx10

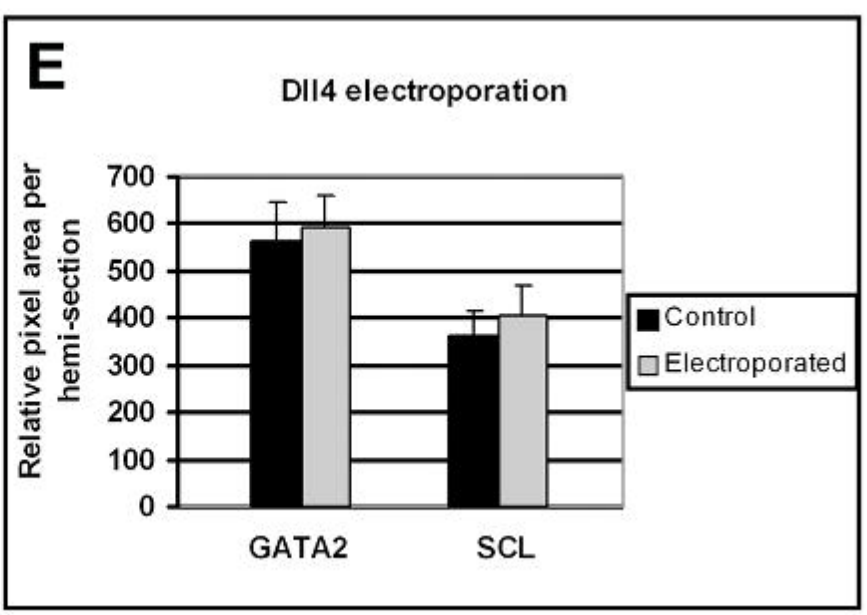

D
Dll4-Myc

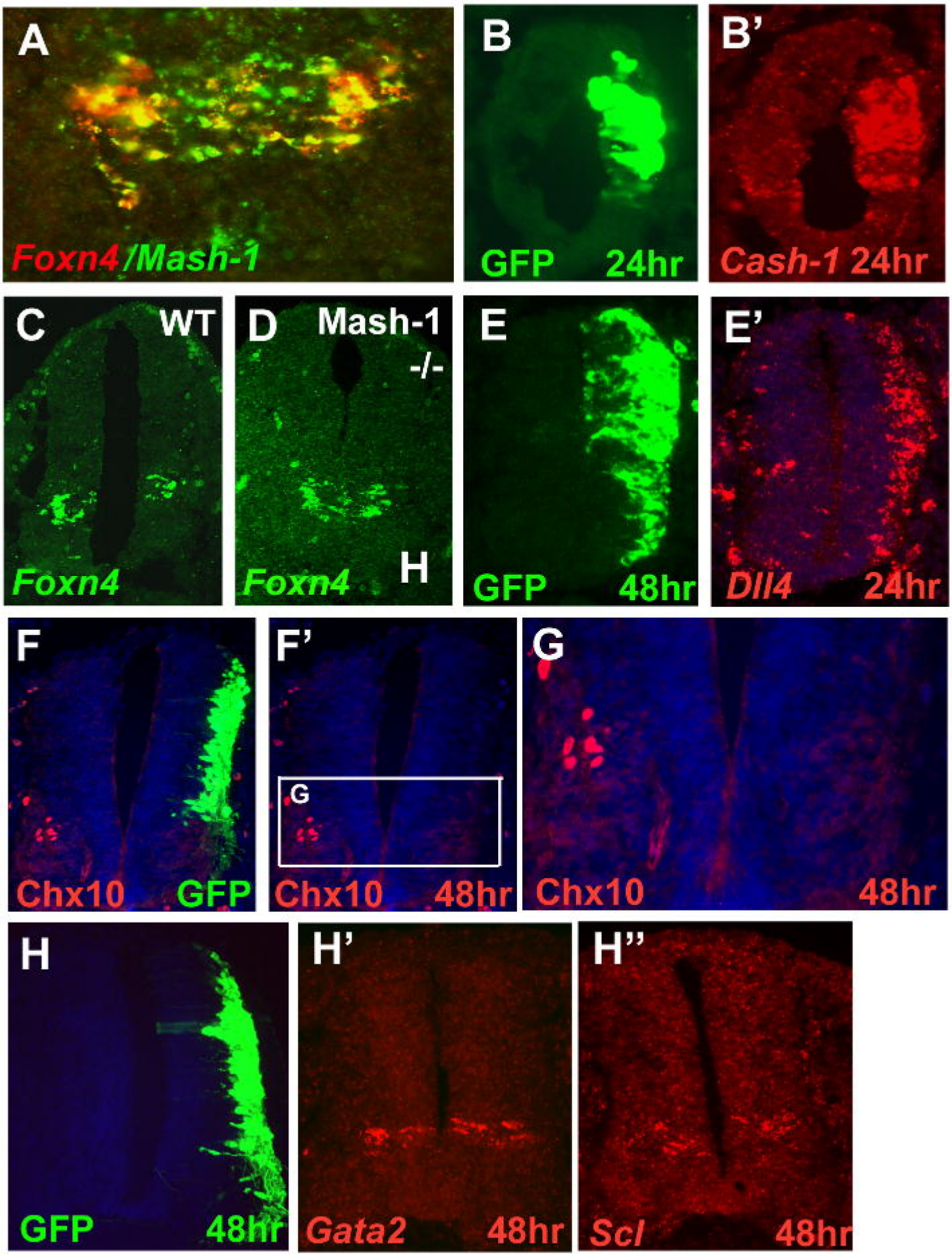

D'
Gata2

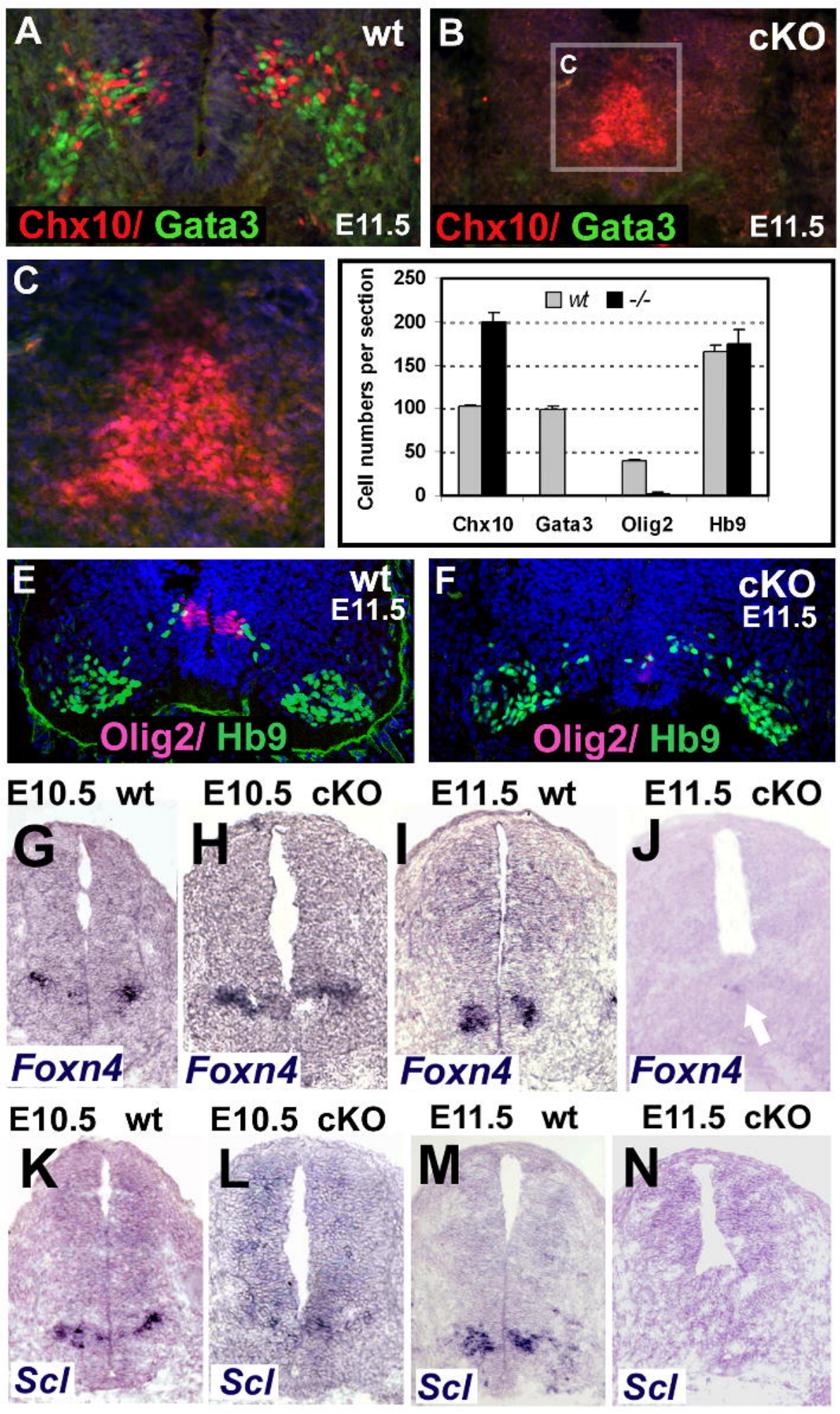

D''
Scl


B
Dll4 electroporation
Cell number per hemi-section
12
10
8
6
4
2
0
CHX10
control
electroporated


C
Dll4-Myc/Chx10


E
Dll4 electroporation
Relative pixel area per hemi-section
700
600
500
400
300
200
100
0
GATA2
SCL
Control
Electroporated


A
Foxn4 /Mash-1
B
GFP 24hr
B'
Cash-1 24hr
C
WT
Foxn4
D
Mash-1
-/-
H
Foxn4
E
GFP 48hr
E'
Dll4 24hr
F
Chx10 GFP
F'
G
Chx10 48hr
G
Chx10 48hr
H
GFP 48hr
H'
Gata2 48hr
H''
Scl 48hr


A
wt
Chx10/ Gata3
E11.5
B
cKO
C
Chx10/ Gata3
E11.5
C
Cell numbers per section
250
200
150
100
50
0
wt
-/-
Chx10
Gata3
Olig2
Hb9
E
wt
E11.5
Olig2/ Hb9
F
cKO
E11.5
Olig2/ Hb9
E10.5 wt
E10.5 cKO
E11.5 wt
E11.5 cKO
G
H
I
J
Foxn4
Foxn4
Foxn4
Foxn4
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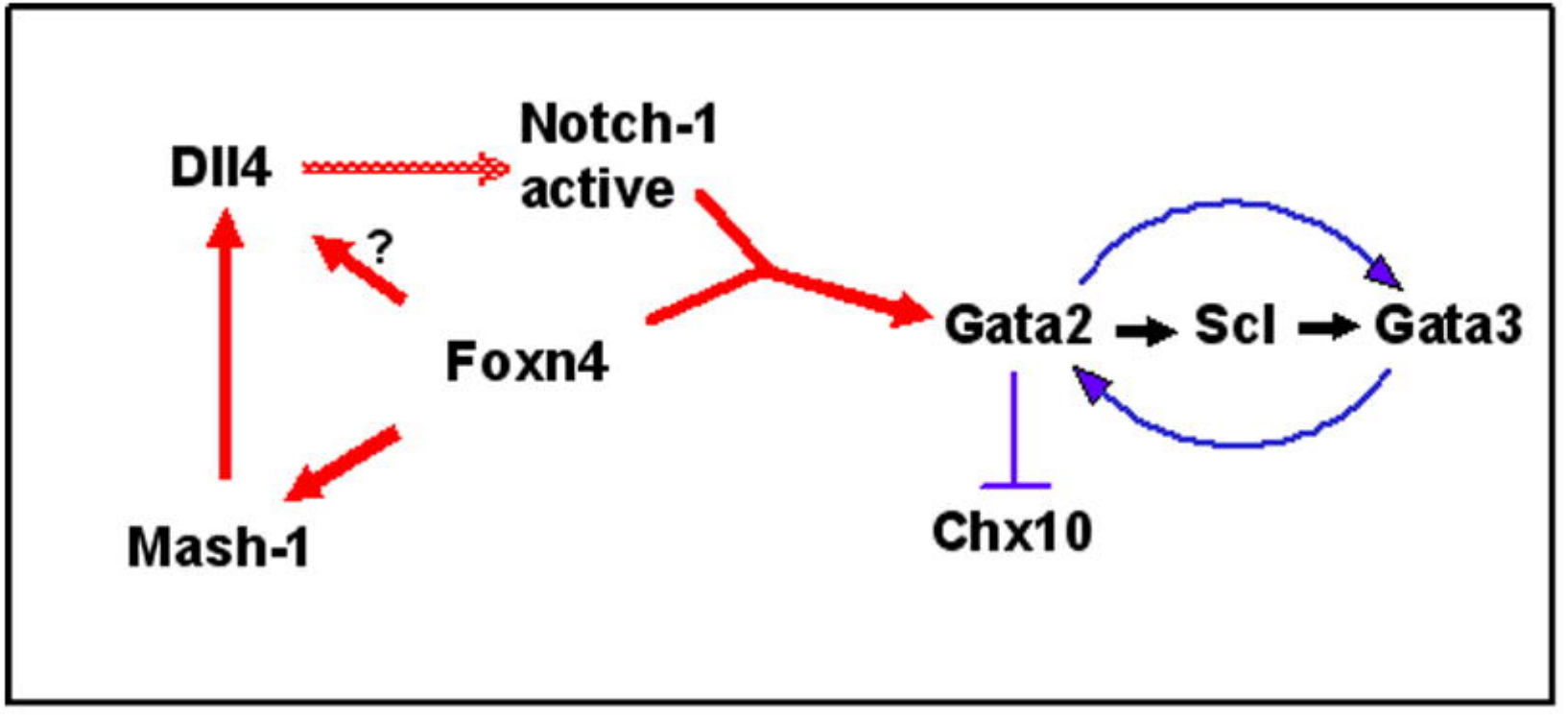
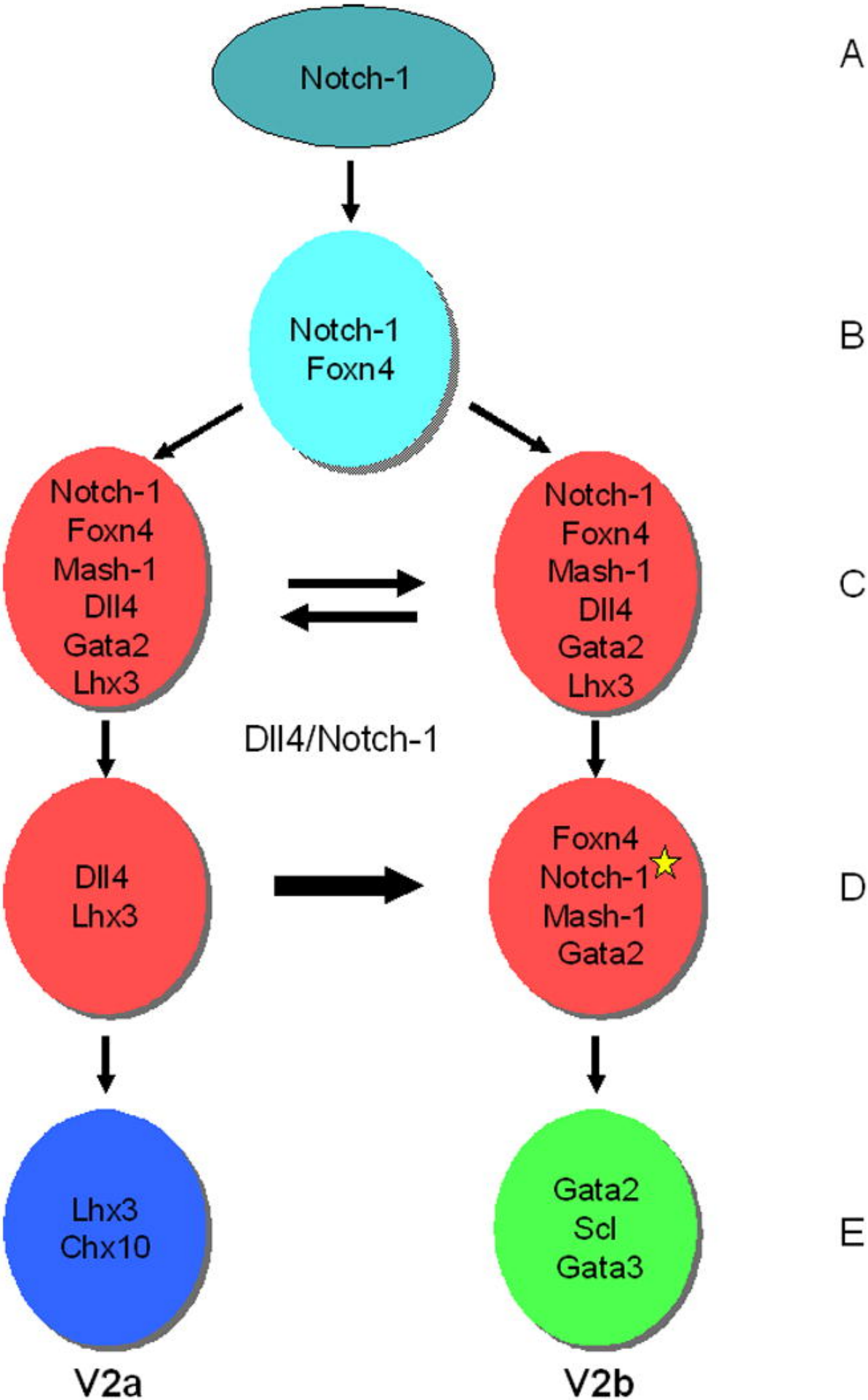
K
L
M
N
Scl
Scl
Scl
Scl


Dll4
Notch-1
active
?
Foxn4
Gata2
Scl
Gata3
Mash-1
Chx10


Notch-1
A
Notch-1
Foxn4
B
Notch-1
Foxn4
Mash-1
Dll4
Gata2
Lhx3
Notch-1
Foxn4
Mash-1
Dll4
Gata2
Lhx3
C
Dll4/Notch-1
Dll4
Lhx3
Foxn4
Notch-1
Mash-1
Gata2
D
Lhx3
Chx10
Gata2
Scl
Gata3
E
V2a
V2b