Development 138, 3199-3211 (2011) doi:10.1242/dev.057281

Regulation of spinal interneuron development by the Olig-related protein Bhlhb5 and Notch signaling

Kaia Skaggs[1,2], Donna M. Martin[2,3] and Bennett G. Novitch[1,2,4,*]

SUMMARY

The neural circuits that control motor activities depend on the spatially and temporally ordered generation of distinct classes of spinal interneurons. Despite the importance of these interneurons, the mechanisms underlying their genesis are poorly understood. Here, we demonstrate that the Olig-related transcription factor Bhlhb5 (recently renamed Bhlhe22) plays two central roles in this process. Our findings suggest that Bhlhb5 repressor activity acts downstream of retinoid signaling and homeodomain proteins to promote the formation of dI6, V1 and V2 interneuron progenitors and their differentiated progeny. In addition, Bhlhb5 is required to organize the spatially restricted expression of the Notch ligands and Fringe proteins that both elicit the formation of the interneuron populations that arise adjacent to Bhlhb5$^+$ cells and influence the global pattern of neuronal differentiation. Through these actions, Bhlhb5 helps transform the spatial information established by morphogen signaling into local cell-cell interactions associated with Notch signaling that control the progression of neurogenesis and extend neuronal diversity within the developing spinal cord.

KEY WORDS: Neurogenesis, Interneurons, Neuronal fate, Notch signaling, Spinal cord, Transcription factors, Mouse, Chick

INTRODUCTION

The control of vertebrate motor behaviors depends on spinal interneuron circuits that relay sensory information from the periphery and modulate motor neuron (MN) activities. This network comprises a diverse array of neurons defined by their expression of certain transcription factors, characteristic settling positions, projections towards different inter- and intrasegmental synaptic targets, neurotransmitter content, and effects on motor outputs (Kiehn, 2006; Goulding, 2009). To achieve the appropriate balance of excitatory and inhibitory inputs, each of these interneuron classes is generated on a precise developmental schedule and in appropriate numbers. Although progress has been made in identifying the determinants for individual interneuron subtypes (Goulding and Pfaff, 2005; Stepien and Arber, 2008), the mechanisms that orchestrate the spatial and temporal progression of their formation within the spinal cord remain poorly defined.

During neurulation, the combined actions of the morphogens sonic hedgehog (Shh) and retinoic acid (RA) induce the spatially restricted expression of a series of class I (induced by RA) and class II (induced by Shh) homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors that together subdivide the neuroepithelium into five discrete progenitor (p) domains along the dorsoventral axis: p0, p1, p2, pMN and p3 (Briscoe and Ericson, 2001; Briscoe and Novitch, 2008). These progenitor groups are defined molecularly by their complement of HD and bHLH proteins, and cellularly by the classes of neurons that they produce: V0-V3 interneurons and MNs (Briscoe and Ericson, 2001; Briscoe and Novitch, 2008). In the case of MN formation, the patterning actions of Shh and RA culminate in the expression of the bHLH transcription factor Olig2, which is uniquely expressed by pMN cells and is essential for MN generation (Rowitch et al., 2002; Briscoe and Novitch, 2008). By contrast, ventral interneurons arise from multiple progenitor populations in the intermediate spinal cord that express varying combinations of the class I HD proteins Pax6, Dbx1 and Dbx2 (Ericson et al., 1997; Pierani et al., 1999; Pierani et al., 2001). It is not clear how the combined activities of these HD proteins are integrated to elicit specific interneuron fates, and whether these progenitor classes similarly depend upon Olig2 equivalents to establish their identities.

Using microarray screening methods to identify genes that are deregulated in the spinal cords of *Olig2*-mutant mice in which MNs are missing and replaced by excessive interneuron production (Rousso et al., 2008) (data not shown), we found that the expression of the Olig-related gene *Bhlhb5* (recently renamed *Bhlhe22*) was significantly increased (see Fig. S1 in the supplementary material). Bhlhb5 expression has been associated with ventral interneuron subtypes such as V2 neurons that are expanded in *Olig2* mutants (see Fig. S1 in the supplementary material) (Rowitch et al., 2002; Brunelli et al., 2003; Liu et al., 2007), raising the possibility that Bhlhb5 might direct these interneuron fates. Bhlhb5 acts as a regulator of neuronal identity in both the retina and neocortex, and is required for the formation of inhibitory interneurons that mediate itch in the dorsal spinal cord (Feng et al., 2006; Liu et al., 2007; Joshi et al., 2008; Ross et al., 2010). However, the contribution of Bhlhb5 to ventral spinal interneuron development has not been determined.

Once formed, neural progenitor domains in the spinal cord typically produce multiple classes of neurons as well as glial cells later in development (Muroyama et al., 2005; Del Barrio et al., 2007; Peng et al., 2007; Hochstim et al., 2008; Rousso et al., 2008). This additional level of neuronal diversification has been attributed to two distinct mechanisms. Different MN subtypes are generated

[1]Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA. [2]Neuroscience Graduate Program, University of Michigan, Ann Arbor, MI 48109, USA. [3]Department of Pediatrics and Communicable Diseases and Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109, USA. [4]Department of Neurobiology, Broad Center of Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA.

*Author for correspondence (bnovitch@ucla.edu)

Accepted 24 May 2011

by the actions of diffusible morphogens on the postmitotic cells, which induce the patterned expression of LIM-HD, Hox and Forkhead domain transcription factors that together define individual MN fates (Sockanathan and Jessell, 1998; Dasen et al., 2008; Rousso et al., 2008). By contrast, V2 interneurons segregate into two distinct classes, excitatory V2a neurons and inhibitory V2b neurons, through cell-cell signaling mediated by Dll4 and Notch receptors (Del Barrio et al., 2007; Peng et al., 2007; Batista et al., 2008; Kimura et al., 2008). Whereas Notch receptors are broadly expressed throughout the spinal cord, Notch ligands and Fringe proteins, which modulate Notch receptor functions, are expressed in a domain-restricted manner (Lindsell et al., 1996; Myat et al., 1996; Yeo and Chitnis, 2007; Rocha et al., 2009; Marklund et al., 2010; Ramos et al., 2010). These observations raise the questions of how these patterns are established, and to what extent the localized expression of Notch ligands contributes to interneuron fate determination and regional control of neurogenesis within the spinal cord.

In this study, we demonstrate that Bhlhb5 (1) acts as a crucial downstream effector of retinoid signaling and Pax6 function, (2) helps direct the formation of dI6, V1 and V2a spinal interneurons, and (3) spatially organizes the regional expression of Notch ligands and Fringe proteins. When mis-expressed with proneural bHLH proteins, Bhlhb5 repressor function directs the ectopic formation of these specific classes of interneurons, whereas acute loss of Bhlhb5 disrupts their development. Modulation of Bhlhb5 function further alters the pattern of Notch ligands and Fringe expression, leading to broad changes in Notch pathway activity and neurogenesis in the intermediate regions of the spinal cord. Together, these findings provide evidence that Bhlhb5 contributes to spinal interneuron differentiation through both direct and indirect mechanisms linked to the Notch signaling pathway.

MATERIALS AND METHODS

Animal preparation and tissue analysis

Fertilized chicken eggs were incubated, staged and electroporated at embryonic day 2 [E2; Hamburger-Hamilton (HH) stages 11-13] or E3 (HH stages 17-18) and analyzed at E5 (HH stage 27) as previously described (Novitch et al., 2001) unless otherwise indicated. *Olig2*$^{GFP/+}$, *Pax6*$^{Sey/+}$ and *Dbx1*$^{lacZ/+}$ mice were maintained and mated for embryo collection as previously described (Ericson et al., 1997; Pierani et al., 2001; Mukouyama et al., 2006).

Immunohistochemistry and in situ hybridization

All embryos were fixed, cryosectioned and processed for antibody staining or in situ hybridization histochemistry as previously described (Novitch et al., 2001; Rousso et al., 2008). Antibodies used were: goat anti-β-galactosidase (Biogenesis, 4600-1409), guinea pig and rabbit anti-chick Bhlhb5 (amino acids 31-50: RSPPGLDLSHPRDRQPSPLAC), guinea pig anti-mouse Bhlhb5 (amino acids 47-63: APPTRERPASSSSPLGC), goat anti-hamster Beta3/Bhlhb5 (Santa Cruz Biotechnology, sc-6045), rat anti-BrdU (BU1/75) (Accurate Chemical, MAS-250p), rabbit anti-chick Chx10 (Ericson et al., 1997), rabbit anti-chick Dbx1 (Pierani et al., 1999; Vue et al., 2007), rabbit anti-chick Dbx2 (Pierani et al., 1999), rabbit anti-human Dll1 (Santa Cruz Biotechnology, sc-9102), goat anti-mouse Dll4 (R&D Systems, AF1389), mouse anti-chick En1 (4G11) [Developmental Studies Hybridoma Bank (DSHB)] (see also Briscoe et al., 2000; Ericson et al., 1997), mouse anti-Evx1 (99.1-3A2) (DSHB) (Pierani et al., 1999), goat anti-human Gata2 (Santa Cruz Biotechnology, sc-267), goat anti-human Gata3 (Santa Cruz Biotechnology, sc-1236), sheep anti-green fluorescent protein (Biogenesis, 4745-1051), mouse anti-rat Isl1 (39.4D5) (DSHB) (Briscoe et al., 2000; Ericson et al., 1997), mouse anti-chick Isl2 (51.4H9) (Briscoe et al., 2000), goat anti-human Jagged1 (Santa Cruz Biotechnology, sc-6011), guinea pig and rabbit anti-mouse Lbx1 (Muller et al., 2005; Muller et al., 2002), mouse anti-chick Lhx3 (67.4E12) (DSHB) (see also Briscoe et al., 2000; Ericson et al., 1997), mouse anti-rat Lhx5 (4F2) (DSHB) (see also Briscoe et al., 2000; Ericson et al., 1997), mouse anti-chick Lmx1b (0.5A5) (DSHB), guinea pig and rabbit anti-Myc epitope tag (Novitch et al., 2001), rat anti-chick NeuroM (Bylund et al., 2003), rabbit anti-chick NeuroM (generous gift of Dr Paul Trainor), rabbit anti-human NF-1A (Active Motif, 39329), guinea pig anti-chick Ngn2 (amino acids 194-211: CEHWPPPRGRFAPPPPPHR) (see also Sandberg et al., 2005), rabbit anti-chick Ngn2 (Zhou et al., 2001), mouse anti-chick Nkx2.2 (74.5A5) (DSHB) (see also Briscoe et al., 2000; Ericson et al., 1997), mouse anti-rat Nkx6.1 (F55A10) (DSHB), rabbit anti-chick Olig2 (Novitch et al., 2001), guinea pig anti-mouse Olig2 (Wichterle et al., 2002), mouse anti-human p27^{Kip1} (BD Biosciences, 610241), mouse anti-chick Pax6 (DSHB) (see also Briscoe et al., 2000; Ericson et al., 1997), mouse anti-chick Pax7 (DSHB) (see also Briscoe et al., 2000; Ericson et al., 1997), guinea pig anti-chick Scl (amino acids 5-19: RPPAPPPPSSDPRDAC), goat anti-human Scl (R&D Systems, AF3360), goat and rabbit anti-human Sox2 (Santa Cruz Biotechnology, sc-17320) (Bylund et al., 2003). Monoclonal antibodies obtained from the DSHB were developed under the auspices of the NICHD and maintained by the University of Iowa.

Digoxigenin-labeled in situ hybridization probes were generated from chick embryonic spinal cord cDNA using PCR and primers directed against the 3′UTR of chick *Scl*, *Dll4*, *Lfng* and *Hes5-2* (see Table S1 in the supplementary material). The mouse *Bhlhb5* in situ probe was generated from EST clone BM950131. mRNA signals were quantified using NIH ImageJ software to measure average pixel intensity for equivalent areas in the intermediate spinal cord on both control and electroporated sides, normalized to background for each image.

Expression constructs

A Bhlhb5 expression vector was generated by PCR subcloning the mouse *Bhlhb5* gene into a Gateway cloning compatible version of the pCIG vector (pCIG-GW) (Megason and McMahon, 2002). Constitutive activator and repressor forms of Bhlhb5 were generated by fusing the Bhlhb5 bHLH domain (amino acids 208-288) in frame to either the Herpes Virus VP16 transactivation domain or the *Drosophila* Engrailed repressor domain, and subcloned into pCIG-GW. The Bhlhb5$^{\Delta b}$ construct was achieved by deletion of amino acids 221-228 through overlap extension PCR mutagenesis. Additional constructs used were either previously described (Novitch et al., 2001; Novitch et al., 2003), or generated by PCR cloning cDNAs into pCIG-GW or RCAS vectors.

shRNA-mediated gene knockdown

Three shRNAs against the endogenous chick *Bhlhb5* sequence (sh1: 5′-TGGAGCATTGCTTACGGAAGA-3′; sh2: 5′-GGAAATCTCTTGAAGGTGAAT-3′; sh3: 5′-TTTAAGCGACTCGCGGAAACA-3′) were cloned into the pRFP-RNAi vector (Das et al., 2006), or into a vector in which the RNAi cassette had been moved into the pCIG backbone. Scrambled shRNA controls were generated by randomizing the nucleotide sequence of each shRNA (sh1 scrambled: 5′-TGCTGGAAAAGTTCGAGTACG-3′; sh2 scrambled: 5′-AGTGGCGAAGTAATTACGATG-3′; sh3 scrambled: 5′-CTTGACAGATATGAGAAGCCC-3′). A non-specific control shRNA (5′-CAGTCGCGTTTGCGACTGG-3′) was based on a sequence lacking similarity to known mammalian and chick genes (Yuan et al., 2007).

RESULTS

Bhlhb5 is expressed in distinct progenitor domains in the developing spinal cord and is retained by subsets of differentiating interneurons

We first generated antibodies to chick and mouse Bhlhb5 to track its expression at key stages of spinal interneuron development. In chick, Bhlhb5 was detected shortly after neural tube closure at embryonic day 2 (E2; HH stage 10) in a group of cells dorsal to the Olig2^{+} pMN domain (Fig. 1A). Bhlhb5 expression coalesced into two distinct progenitor stripes by E3 (HH stages 17-19; Fig. 1B), and a third stripe dorsal to the pMN containing scattered Bhlhb5^{+}

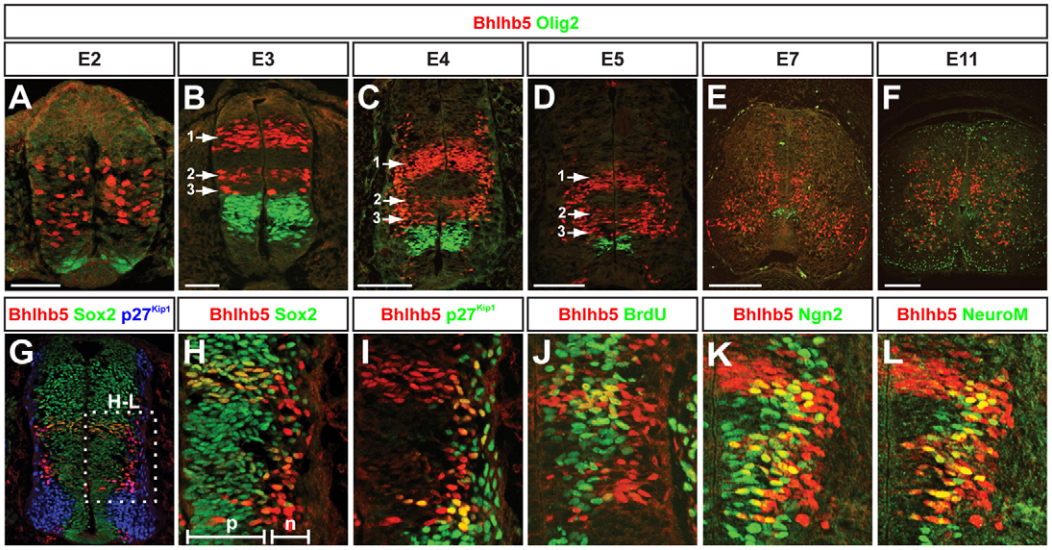

Fig. 1. Bhlhb5 is complementary to Olig2 in spinal cord progenitors and is retained by subsets of differentiating neurons. (**A-F**) Co-staining analysis of Bhlhb5 and Olig2 expression in the developing chick spinal cord. Arrows and numbers indicate distinct progenitor stripes of Bhlhb5 expression. Scale bars: 50 μm in A,B; 100 μm in C,D; 500 μm in E,F. (**G-J**) Bhlhb5 is expressed by both Sox2$^+$ progenitors (p) and p27^{Kip1+} postmitotic neurons (n) that incorporate BrdU. Dotted box indicates area shown in H-L. (**K**,**L**) Bhlhb5 transiently overlaps with both Ngn2 and NeuroM.

cells became detectable between E3 and E5 (HH stages 18-27; Fig. 1B-D). Bhlhb5 staining varied within these progenitors, with the highest expression seen in the dorsal-most stripe and weakest in the middle stripe (Fig. 1B-D). Bhlhb5 and Olig2 remained exclusive of one another throughout early embryogenesis (Fig. 1A-F). At gliogenic stages such as E11 (HH stage 37) and later, nearly all Bhlhb5$^+$ cells expressed the neuronal marker NeuN, though a few Bhlhb5$^+$ cells lacked NeuN and instead expressed the astrocyte progenitor marker NF1A (data not shown) (Deneen et al., 2006). Thus, Bhlhb5 appears to mark several populations of interneuron progenitors and differentiated neurons in the intermediate spinal cord, as well as a small number of astrocyte progenitors, reminiscent of the sequential expression of Olig2 in progenitors that give rise to MNs and then oligodendrocytes (Rowitch et al., 2002).

Previous reports have suggested that Bhlhb5 is confined to postmitotic cells in both the retina and spinal cord (Feng et al., 2006; Liu et al., 2007; Ross et al., 2010). However, in the E4 chick Bhlhb5 extensively overlapped with the neural progenitor marker Sox2 (Fig. 1G,H) (Bylund et al., 2003; Graham et al., 2003), and these cells were readily labeled with a 30-minute pulse of BrdU (Fig. 1J). Bhlhb5 was also present in actively differentiating neurons marked by the proneural bHLH proteins Neurogenin2 (Ngn2) and NeuroM, and postmitotic cells expressing the cyclin-dependent kinase inhibitor p27^{Kip1} (Fig. 1G,I,K,L). Similar results were observed in the E9.5-10.5 mouse spinal cord, where low levels of Bhlhb5 protein were detected in Sox2$^+$ and Ki-67$^+$ progenitors and higher levels in differentiated cells (see Fig. S2A-E in the supplementary material), indicating that in both species Bhlhb5 is initially expressed by dividing progenitors and then maintained by specific neuronal populations.

We next mapped Bhlhb5 expression with respect to HD transcription factors that demarcate discrete progenitor domains in the spinal cord. Bhlhb5 was closely associated with Pax6 expression in several interneuron progenitor domains from pdI6 dorsally to p2 ventrally (Fig. 2A,N) (Ericson et al., 1997). The Pax6 region is subdivided by Dbx2, which spans the pdI6 to p1 domains, and Dbx1, which is confined to the p0 domain (Pierani et al., 1999; Pierani et al., 2001). Bhlhb5 was present in the dorsal- and ventral-most Dbx2$^+$ progenitors, but was not co-expressed with Dbx1 (Fig. 2B,C,N). The ventral extent of Bhlhb5 expression coincided with Nkx6.1$^+$ p2 interneuron progenitors, but did not extend into the Olig2$^+$ pMN domain (Fig. 1C, Fig. 2D,N).

By E5 (HH stage 27), Bhlhb5 was prominently associated with interneurons that expressed the LIM-HD proteins Lhx1 and Lhx5 (Fig. 2F) and markers of specific interneuron subclasses. Bhlhb5 was present in the ventral-most group of Lbx1$^+$ cells, presumed to be dI6 interneurons, yet absent from more dorsal Lbx1$^+$ cells such as Lmx1b$^+$ dI5 interneurons (Fig. 2G,H) (Gross et al., 2002; Muller et al., 2002). Bhlhb5 was also missing from Evx1$^+$ V0 interneurons (Fig. 2I), but was expressed by other ventral interneuron populations including a subset of En1$^+$ V1 interneurons and Chx10$^+$ V2a interneurons (Fig. 2J,K) (Briscoe et al., 2000; Del Barrio et al., 2007; Peng et al., 2007). However, Bhlhb5 was notably absent from most Gata3$^+$ and Scl (Tal1)$^+$ V2b interneurons (Fig. 2L,M; see Fig. S3 in the supplementary material). Bhlhb5 was expressed earlier than the previously characterized markers of V2 neurons such as FoxN4, Gata2 and Lhx3 (see Fig. S3 in the supplementary material) (Li et al., 2005; Del Barrio et al., 2007; Peng et al., 2007), suggesting that its expression precedes their subdivision into the V2a and V2b classes. Bhlhb5 expression was also seen in a subset of Nkx2.2$^+$ V3 interneurons, but not in differentiated MNs (Fig. 2E; data not shown). With the exception of the V3 interneuron expression, Bhlhb5 was expressed in a very similar pattern in the mouse, indicating an evolutionarily conserved association of Bhlhb5 with dI6, V1 and V2a progenitors and their differentiated progeny (Fig. 2N; see Fig. S2F-M in the supplementary material).

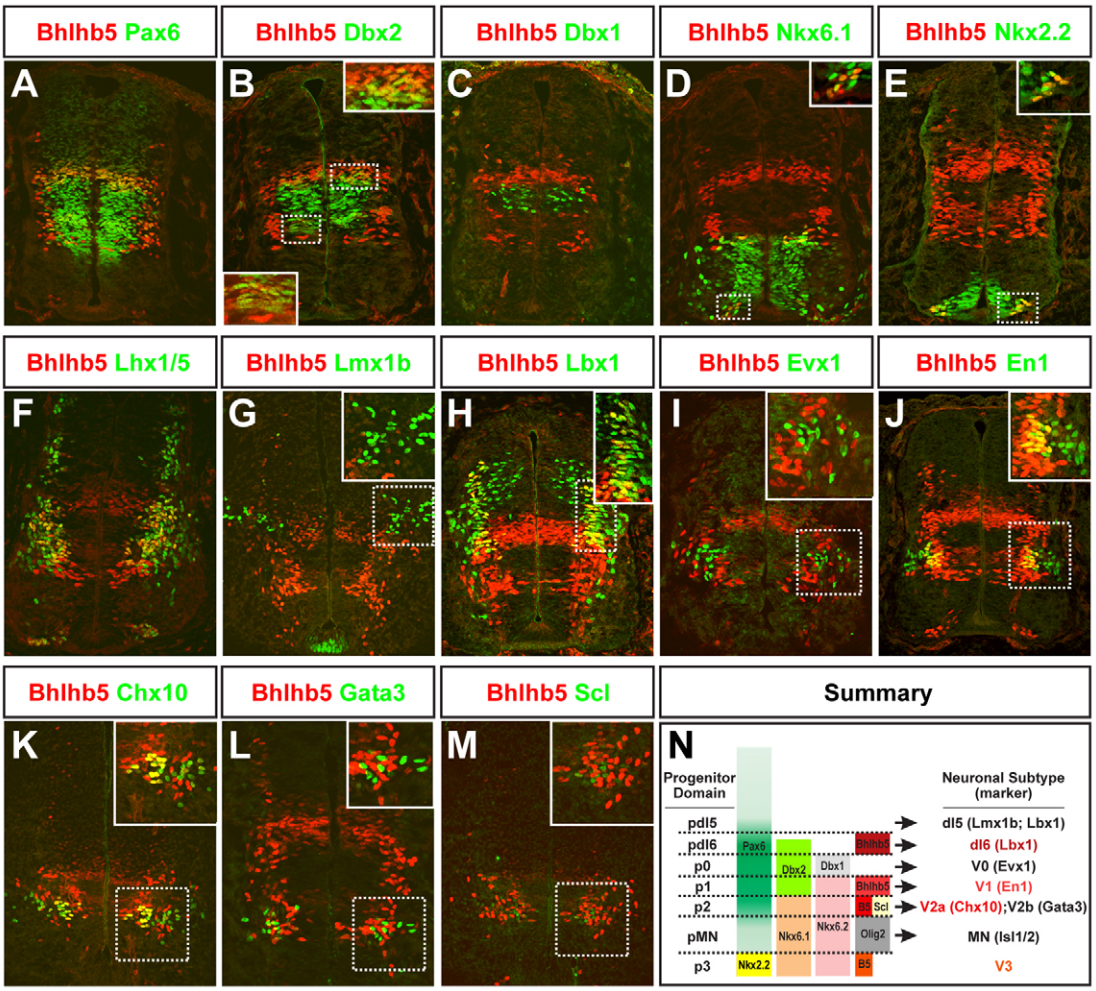

Fig. 2. Bhlhb5 is selectively expressed by dI6, V1 and V2a progenitors and their interneuron progeny. (**A-E**) Antibody co-staining of Bhlhb5 and HD proteins that demarcate the boundaries of progenitors in the E4 chick spinal cord. (**F-M**) At E5, Bhlhb5 is expressed by several distinct groups of interneurons. Dotted boxes in B,D,G-M indicate regions enlarged in insets. (**N**) Summary of the expression pattern of Bhlhb5 and its association with specific classes of interneurons.

Bhlhb5 expression depends on retinoid signaling and Pax6 activity, and is spatially confined by both Olig2 and Dbx1

Retinoid signaling and Pax6 transcriptional activity provide the major stimulus for interneuron development in the intermediate spinal cord (Ericson et al., 1997; Pierani et al., 1999). To examine the relationship between these factors and Bhlhb5, we used in ovo electroporation approaches in chick to mis-express a dominant-negative form of the RA receptor α (dnRAR) that blocks RA signaling in vivo (Novitch et al., 2003). This construct potently suppressed Pax6 and Bhlhb5 (Fig. 3A,B), indicating that both factors are retinoid dependent. Co-electroporation of dnRAR along with Pax6 expression constructs restored Bhlhb5 levels (Fig. 3C,D), suggesting that Pax6 acts downstream of retinoid signaling to promote Bhlhb5 expression. Consistent with this conclusion, Bhlhb5 was reduced in *Pax6*$^{Sey/Sey}$-mutant mice, and this loss closely mirrored the interneuron deficit seen in these animals (Fig. 3E,F) (Burrill et al., 1997; Ericson et al., 1997; Osumi et al., 1997).

The progenitor domains in the spinal cord are established by cross-repressive interactions of class I and class II transcription factors that act downstream of RA and Shh signaling (Briscoe and Novitch, 2008). Olig2, for example, opposes both Nkx2.2 and Irx3 to define the borders of the p3, pMN and p2 progenitor domains (Mizuguchi et al., 2001; Novitch et al., 2001). We sought to determine whether similar interactions occur between Bhlhb5 and the proteins that define its neighboring progenitor domains. Olig2 mis-expression in the chick strongly repressed Bhlhb5, whereas Bhlhb5 expanded ventrally in the spinal cord of *Olig2*-mutant mice (Fig. 3G-J; see Fig. S1A-J in the supplementary material). When Bhlhb5 was mis-expressed, Olig2 was decreased (Fig. 3M,Q), suggesting that cross-repressive interactions between Olig2 and Bhlhb5 help to maintain the boundary between the p2 and pMN domains.

To account for the exclusion of Bhlhb5 from p0 progenitors and V0 interneurons, we next examined *Dbx1*$^{lacZ/lacZ}$ knockout mice, which lack these cells (Pierani et al., 2001). In *Dbx1*$^{lacZ/+}$ controls, Bhlhb5 expression was excluded from most β-galactosidase (β-Gal)$^{+}$ p0 progenitors and their derivatives (Fig. 3K). However, in *Dbx1*$^{lacZ/lacZ}$ mutants, Bhlhb5 expression expanded into many of the β-Gal^{+} cells (Fig. 3L). The interactions between Dbx1 and Bhlhb5 were reciprocal, as Bhlhb5 mis-expression potently suppressed Dbx1 and to a much lesser extent Dbx2 (Fig. 3O,P). However, Bhlhb5 mis-expression did not alter other class I HD proteins such as Pax6, Pax7 or Irx3, revealing specificity to its

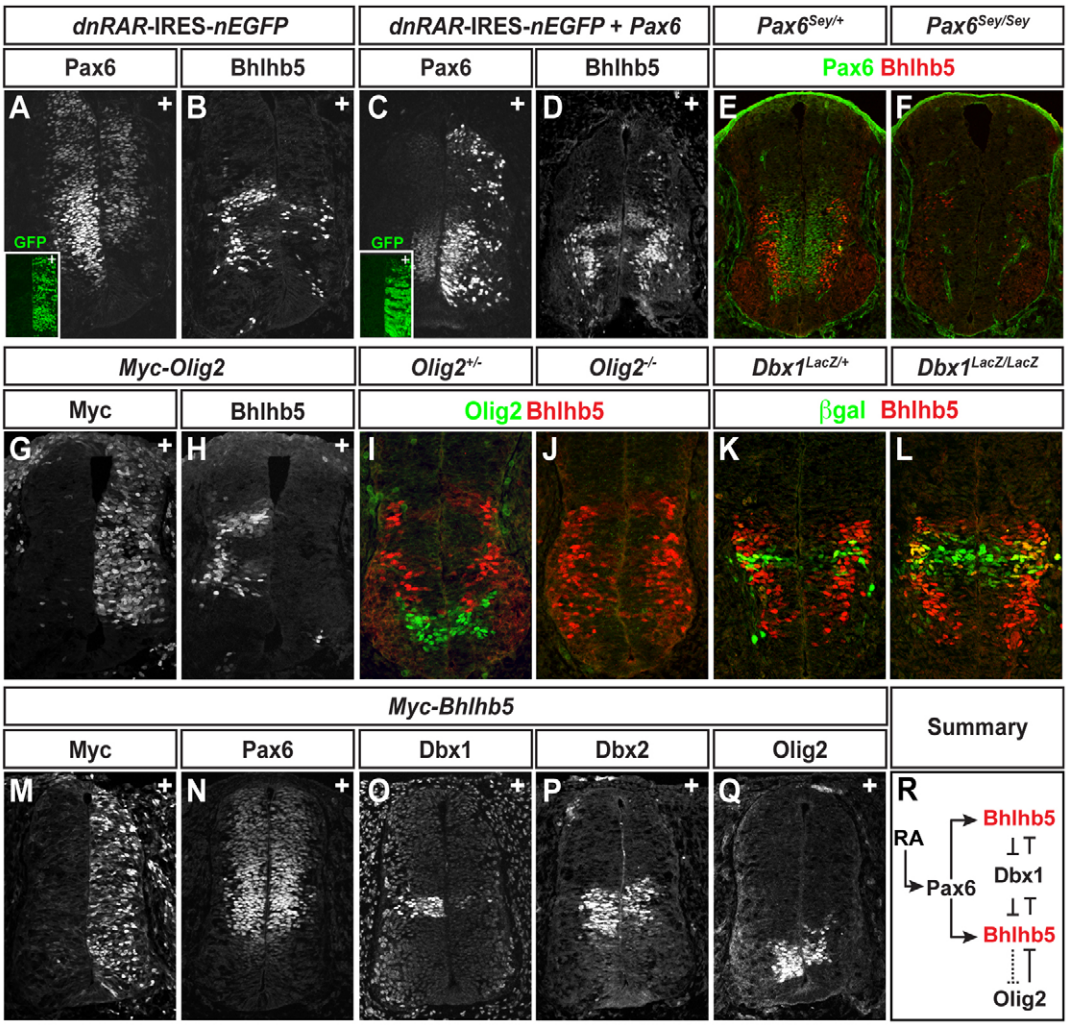

Fig. 3. Bhlhb5 depends on retinoid signaling and Pax6 activity, and is spatially restricted by cross-repressive interactions with Olig2 and Dbx1. (**A-D**) Mis-expression of a dominant-negative retinoic acid (RA) receptor represses both Pax6 and Bhlhb5. Bhlhb5 is restored by co-electroporation of Pax6 expression constructs. (**E**,**F**) Bhlhb5 is lost in E10.5 *Pax6*$^{Sey/Sey}$-mutant mice. (**G**,**H**) Olig2 mis-expression represses Bhlhb5. (**I**,**J**) Bhlhb5 expands ventrally in E10.5 *Olig2*-mutant mice. (**K**,**L**) Bhlhb5 expands into the β-galactosidase (β-gal)$^+$ p0 progenitor domain in E11.5 *Dbx1*$^{lacZ/lacZ}$-mutant mice. (**M-Q**) Mis-expression of a Myc-tagged form of Bhlhb5 does not alter Pax6 but does suppress Dbx1 and, to a lesser extent, Olig2 and Dbx2. Plus sign indicates the transfected side of the spinal cord. (**R**) Summary of the upstream signals and cross-repressive interactions that regulate Bhlhb5 expression.

actions (Fig. 3N; data not shown). Thus, Bhlhb5 appears to be promoted by both retinoid signaling and Pax6 function, and restricted from specific progenitor domains by repressive interactions with Dbx1 and Olig2 (Fig. 3R).

Mis-expression of Bhlhb5 with neurogenins leads to ectopic generation of dI6, V1 and V2a interneurons and suppresses the development of other neuronal classes

The selective expression of Bhlhb5 in dI6, V1 and V2a progenitors and the correlation of changes of Bhlhb5 expression with altered interneuron development in *Olig2* and *Pax6* mutants suggest that Bhlhb5 might act as a determinant of these fates. Supporting this prediction, mis-expression of Bhlhb5 with an IRES-nuclear EGFP (nEGFP) reporter throughout the chick spinal cord led to the formation of a small number of ectopic Chx10$^+$ and Lhx3$^+$ V2a interneurons, and Lbx1$^+$ cells presumed to be dI6 interneurons owing to their ventral position and lack of Lmx1b expression (Fig. 4A-C,T,U,W; see Fig. S4A-D in the supplementary material). By contrast, other cell populations such as Evx1$^+$ V0, En1$^+$ V1 and Gata3$^+$/Scl$^+$ V2b interneurons were significantly reduced (Fig. 4D,E,T,V; see Fig. S4E-G in the supplementary material).

The net loss of interneuron subtypes exceeded the gains seen in the V2a and dI6 populations, raising the possibility that Bhlhb5 mis-expression might be either toxic to cells or capable of impairing neuronal differentiation. Bhlhb5 mis-expression did not increase apoptotic death measured by activated caspase 3 antibody staining (data not shown), but did reduce Ngn2 expression and the appearance of postmitotic neurons by ~10-20% (Fig. 7X). This inhibition might reflect the previously observed ability of high levels of Bhlhb5 to block interactions between proneural bHLH transcription factors and their E protein DNA binding partners in an Id protein-like manner (Peyton et al., 1996; Xu et al., 2002; Ruzinova and Benezra, 2003). However, an Id-like function for endogenous Bhlhb5 seems unlikely as it was normally associated with differentiating neurons (Fig. 1I,K,L).

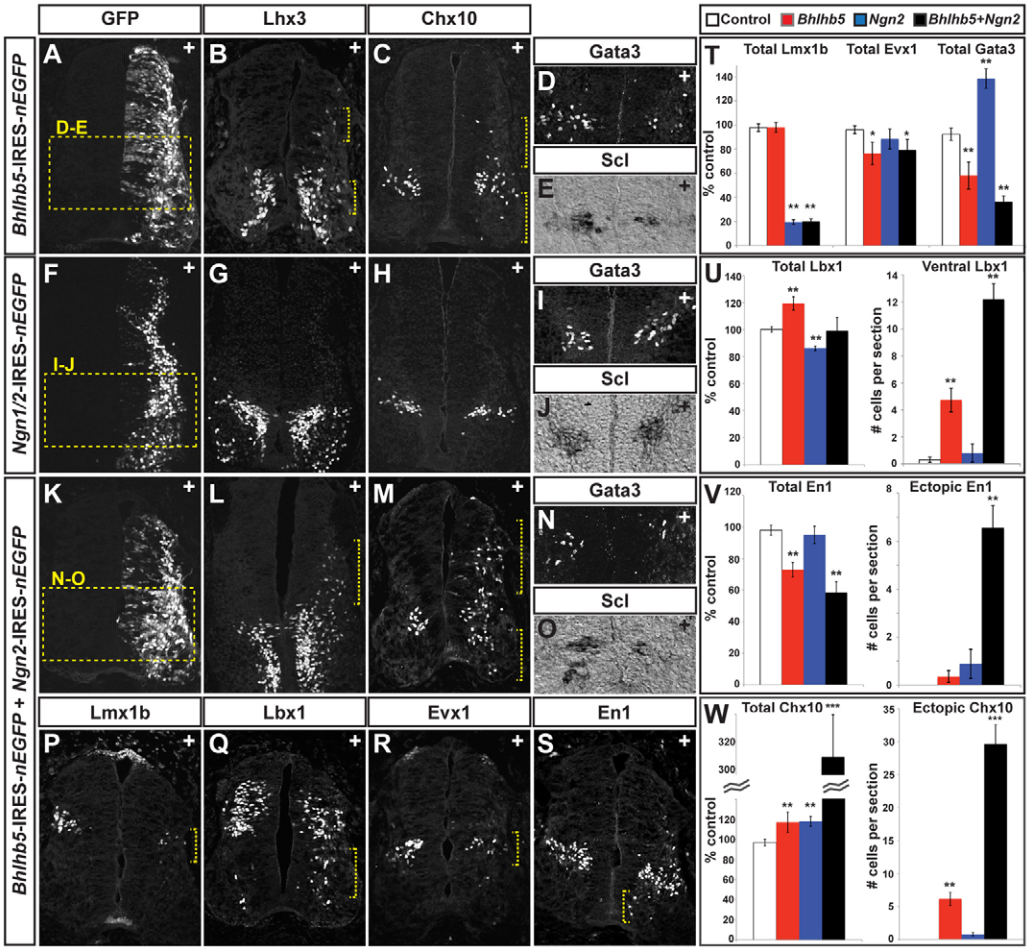

Fig. 4. Mis-expression of Bhlhb5 and Ngn leads to ectopic generation of dI6, V1 and V2a interneurons and the suppression of other interneuron classes. (**A-E**) Bhlhb5 mis-expression mildly expands V2a interneuron formation (brackets), and reduces V2b interneurons. (**F-J**) Ngn2 mis-expression promotes neuronal differentiation, but does not alter V2a or V2b fates. (**K-S**) The combined mis-expression of Bhlhb5 with Ngn2 produces ectopic V2a interneurons throughout the spinal cord (brackets) and Lbx1$^+$ cells in the ventral spinal cord but decreases the formation of other interneuron classes. (**T-W**) Quantification of neurons formed under the conditions described above. Total cell numbers are represented as percentage control based on the ratio of cell numbers on the experimental side of the spinal cord compared with the non-electroporated side. Control electroporations refers to embryos transfected with an empty vector. Ectopic En1 and Chx10 cells were defined as cells dorsal or ventral to their normal position on the non-electroporated side of the spinal cord. Ventral Lbx1 cells were scored as the number of Lbx1 cells below the midpoint of the dorsoventral axis. Mean ± s.e.m. plotted are representative of multiple sections collected from at least ten successfully electroporated embryos for each experimental condition. Statistical significance was determined by Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001. Plus sign indicates the transfected side of the spinal cord.

To restore the balance between Bhlhb5 and proneural proteins in these gain-of-function assays, we co-expressed Bhlhb5 with Ngn1 or Ngn2 (collectively referred to hereafter as Ngn) which produced numerous V2a interneurons throughout the spinal cord (Fig. 4K-M,W; see Fig. S4CC in the supplementary material). Under these conditions, ectopic dI6-like and V1 interneurons in the ventral spinal cord were also enhanced though the numbers of these cells in their endogenous positions were typically reduced (Fig. 4Q,S,U,V; see Fig. S4BB,EE in the supplementary material). Nearly all of the ectopic cells expressed the GFP transfection marker, indicating that these effects are primarily due to the cell-autonomous actions of Bhlhb5 and Ngn (data not shown). Bhlhb5 and Ngn co-expression consistently inhibited the formation of other ventral interneuron subtypes such as V0 and V2b interneurons, much like that seen with Bhlhb5 mis-expression alone (Fig. 4D,E,N,O,R,T; see Fig. S4FF,GG in the supplementary material), suggesting that the loss of endogenous dI6, V0, V1 and V2b interneurons might result from a conversion of these cells into V2a interneurons. This seems to be the case for V2b interneurons as Bhlhb5 mis-expression strongly suppressed the early V2b fate determinants Scl and Foxn4 at the same time that V2a markers were increased (Fig. 4B,C,E; see Fig. S5C in the supplementary material).

Mis-expression of Ngn by itself promoted neuronal differentiation, but did not alter the assignment of most specific interneuron fates with the exception of Lmx1b$^+$ Lbx1$^+$ dI5 interneurons, which were suppressed regardless of whether Bhlhb5 was present (Fig. 4T; see Fig. S4K,DD in the supplementary material). Although Ngn mis-expression slightly increased the number of Chx10$^+$ V2a and Gata3$^+$ V2b neurons formed, it did not change the dorsoventral position at which these cells were generated (Fig. 4F-J,T-W; see Fig. S4H-N and Fig. S5D in the supplementary material). The observed effects of Bhlhb5 on interneuron fate selection were also not

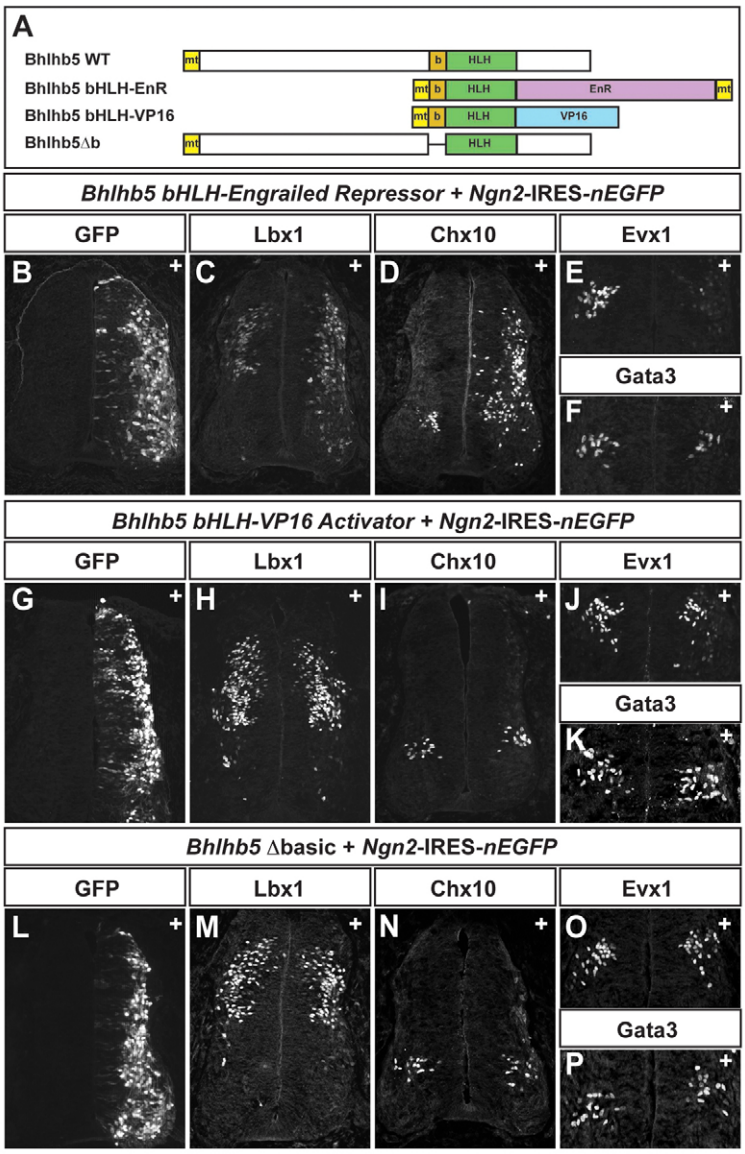

Fig. 5. Bhlhb5 directs specific neuronal fates through its actions as a transcriptional repressor. (**A**) Illustration of the modified forms of Bhlhb5 used to test transcriptional activity. b, basic DNA binding region; HLH, helix-loop-helix dimerization motif; mt, myc epitope tag. (**B-P**) Co-expression of Bhlhb5 bHLH-EnR and Ngn2 recapitulates the activities of full-length Bhlhb5 and Ngn2 whereas the equivalent co-expression of Bhlhb5 bHLH-VP16 or Bhlhb5$^{\Delta b}$ and Ngn2 has little effect on neuronal fates. Plus sign indicates the transfected side of the spinal cord.

recapitulated by mis-expression of Id1 (see Fig. S4O-U,HH-MM in the supplementary material) indicating that the actions of Bhlhb5 are distinct from those exhibited by Id proteins. Collectively, these results indicate that the combined transcriptional activities of Bhlhb5 and Ngn are sufficient to direct the differentiation of V2a interneurons, and to a lesser extent dI6 and V1 interneurons, and to suppress the formation of other neuronal classes.

Bhlhb5 promotes dI6 and V2a interneuron formation through its transcriptional repressor activity

Members of the Olig gene family direct specific neuronal fates through their function as transcriptional repressors (Novitch et al., 2001; Muller et al., 2005), raising the question of whether Bhlhb5 acts in a similar manner. We therefore created dominant repressor and activator forms of Bhlhb5 by fusing its bHLH DNA binding region to either the transcriptional repressor domain from the *Drosophila* Engrailed protein (EnR) or the Herpes virus VP16 transcriptional activation domain (Fig. 5A) (Novitch et al., 2001). To confirm further that Bhlhb5 requires DNA binding activity, we created a mutant form of full-length Bhlhb5 lacking the basic region (Bhlhb5$^{\Delta b}$). When co-expressed with Ngn2, Bhlhb5-EnR produced a large number of ectopic V2a and dI6-like interneurons throughout the spinal cord, and potently suppressed the formation of V0 and V2b interneuron much like the full-length Bhlhb5 protein (Fig. 5B-F). By contrast, neither Bhlhb5-VP16 nor Bhlhb5$^{\Delta b}$ significantly affected the pattern of interneuron formation aside from minor reductions in cell numbers (Fig. 5G-P; data not shown). Thus, the ability of Bhlhb5 to regulate spinal interneuron development depends on its ability to bind and repress specific DNA targets.

Bhlhb5 knockdown disrupts the development of multiple interneuron classes

Next, we sought to determine whether endogenous Bhlhb5 function is required for interneuron generation using gene knockdown approaches in chick embryos. We used a plasmid vector to deliver three different short hairpin RNA (shRNA) constructs against the untranslated regions of the chick *Bhlhb5* gene (Fig. 6A) or scrambled shRNA controls. Whether expressed individually or in combination, the Bhlhb5 shRNAs decreased the number of cells expressing

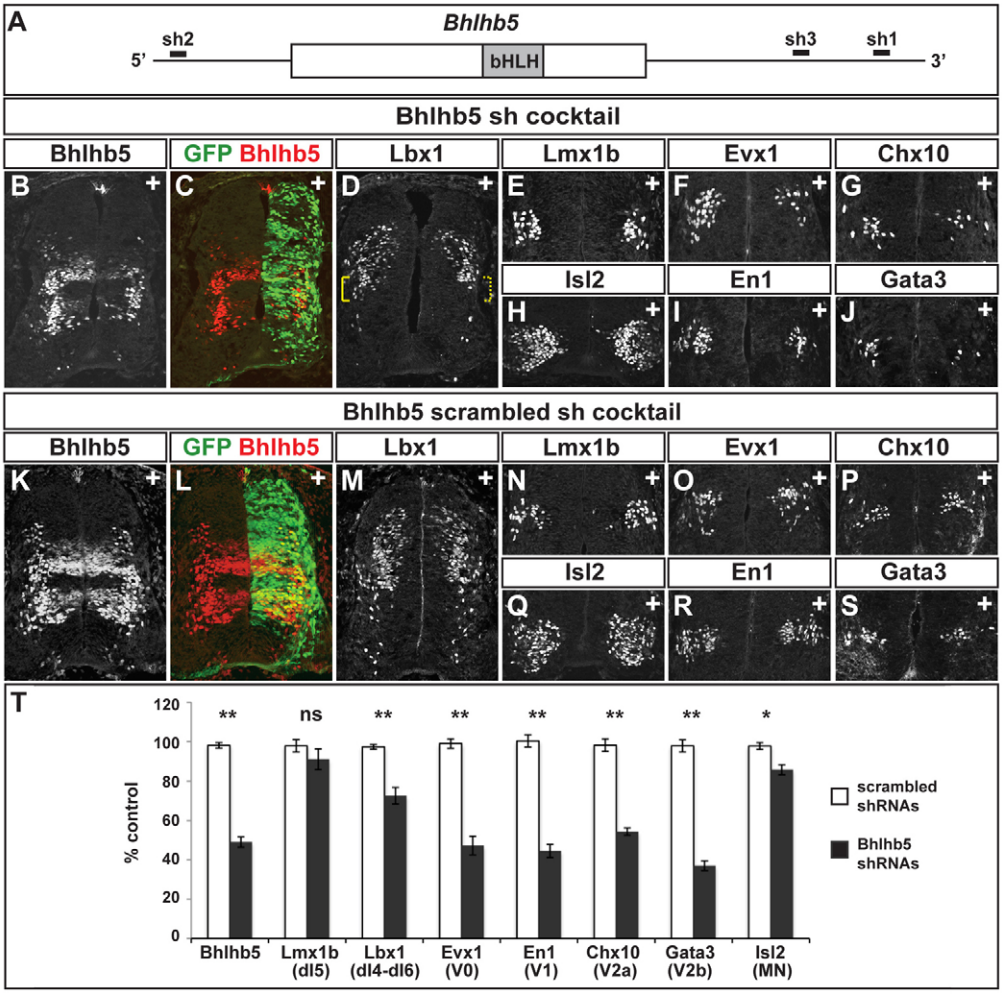

Fig. 6. Loss of Bhlhb5 function reduces the formation of dI6, V1 and V2a interneurons as well as adjacent interneuron populations. (**A**) Schematic showing the location of the three short hairpin targets (sh1, sh2 and sh3) against chick *Bhlhb5*. (**B-J**) Antibody staining of chick spinal cords electroporated at E3 with a cocktail of the three Bhlhb5 shRNAs. Brackets in D indicate the position of dI6 neurons on the non-transfected control and transfected sides of the spinal cord. (**K-S**) The equivalent electroporation using a cocktail of scrambled shRNAs has no effect on interneuron development. (**T**) Graph of the number of cells expressing each neuronal subtype marker expressed as a percentage of that seen on the unelectroporated control side of the spinal cord. Mean ± s.e.m. plotted are representative of multiple sections collected from at least ten successfully electroporated embryos. Statistical significance was determined by Student's *t*-test: **P*<0.05; ***P*<0.01; ns, not significant. Plus sign indicates the transfected side of the spinal cord.

Bhlhb5 by ~50%, and the protein that remained was reduced compared with the non-transfected side of the spinal cord (Fig. 6B,C,T; see Fig. S6H,I,O,P,V,W in the supplementary material).

Although Bhlhb5 mis-expression can alter the dorsoventral patterning of the spinal cord (Fig. 3), Bhlhb5 knockdown did not lead to any significant change in the establishment of interneuron progenitor domains (Fig. 7W, see Fig. S5G,H in the supplementary material; data not shown), suggesting that its contributions might be redundant with those mediated by class I and II HD proteins. Despite the seemingly normal formation of interneuron progenitors, Bhlhb5 loss nevertheless led to a ~50% decrease in all of the neuronal subtypes with which it is associated, including the ventral-most Lbx1$^+$ dI6-like cells, as well as En1$^+$ V1 and Chx10$^+$ V2a interneurons (Fig. 6D,G,J,T).

Intriguingly, Bhlhb5 knockdown also reduced the generation of Evx1$^+$ V0 interneurons and Gata3$^+$ V2b interneurons (Fig. 6F,J,T; see Fig. S5F in the supplementary material), which lack Bhlhb5 (Fig. 2I,L), to an extent that was comparable to that seen with dI6, V1 and V2a interneurons. The effects of Bhlhb5 loss were, nevertheless, selective, as there was only a small (~10%) decrease in Isl2$^+$ MN production and no change in dI5 neuron formation (Fig. 6E,H,T). Similar results were achieved with the individual expression of the three *Bhlhb5* shRNA constructs but not with scrambled shRNA sequences or a control shRNA vector (Fig. 6K-T; see Fig. S6A-BB in the supplementary material), indicating that these results are specific to the reduction of *Bhlhb5* and unlikely to result from off-target effects. Moreover, we did not observe any significant increase in apoptotic cell death that could account for these results (data not shown), and the suppressive effects of Bhlhb5 knockdown were rescued by the mis-expression of a mouse Bhlhb5 cDNA lacking the shRNA binding sites (see Fig. S6CC-II in the supplementary material). Together with our mis-expression results, these data suggest that cell-autonomous functions of Bhlhb5 are required for the formation of dI6, V1 and V2a interneurons, whereas non-cell-autonomous functions influence the generation of interneuron populations that form adjacent to Bhlhb5$^+$ cells.

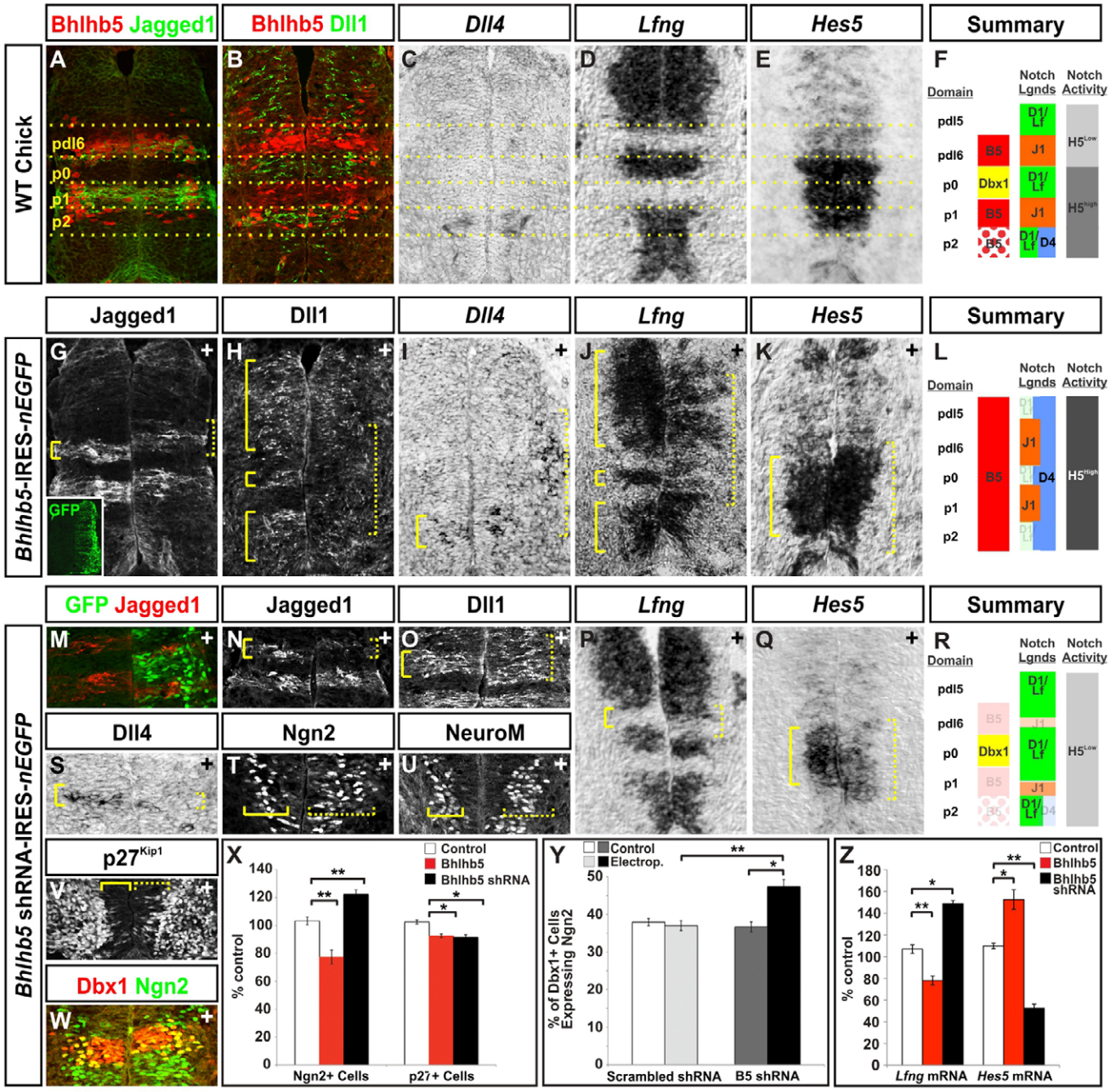

Fig. 7. Bhlhb5 regulates the pattern of Notch ligands, *Lfng* and neurogenesis in the spinal cord. (**A-E**) Notch ligands and *Lfng* are expressed in a domain-restricted manner that coincides with the presence (Jag1 and *Dll4*) or absence (Dll1 and *Lfng*) of Bhlhb5 (A-D). *Hes5* mRNA provides a readout for ongoing Notch pathway activity in the intermediate spinal cord (E). The approximate boundaries of neural progenitor domains are indicated by the dotted lines. (**F**) Summary of the domain relationship between Bhlhb5, HD transcription factors, Notch ligands and Notch pathway activity under control conditions. (**G-L**) Mis-expression of Bhlhb5 represses both Dll1 and *Lfng*, and expands Jagged1 and *Dll4*. Under these conditions, *Hes5* levels increase and expand dorsally. Brackets indicate the domains of expression on the non-transfected control and transfected sides of the spinal cord. (**M-V**) Bhlhb5 knockdown reduces Jagged1 and *Dll4*, and expands Dll1 and *Lfng* particularly in the pdl6 region. *Hes5* levels decline whereas the density of differentiating neurons expressing Ngn2, NeuroM and $p27^{Kip1}$ increases. (**W**) Although Bhlhb5 knockdown does not inhibit the formation of $Dbx1^{+}$ p0 cells, Ngn2 is nevertheless increased in this region. (**X**) Quantification of the effects of Bhlhb5 mis-expression and knockdown on Ngn2 expression and the formation of $p27^{Kip1+}$ neurons. Experimental manipulations were compared with cells expressing the indicated markers on the contralateral control side of the spinal cord. An empty expression vector control was also included to account for non-specific electroporation effects. Mean ± s.e.m. plotted are representative of multiple sections collected from at least ten successfully electroporated embryos for each experimental condition tested and plotted as percentage of control. Statistical significance was determined by Student's *t*-test: $*P<0.02$; $**P<0.002$. (**Y**) Comparison of the ratio of $Dbx1^{+}$ p0 cells expressing Ngn2 on the electroporated side of the spinal cord versus the contralateral control side. $*P<0.001$ (paired samples); $**P<0.002$ (independent samples). (**Z**) Quantification of *Lfng* and *Hes5* mRNA expression in the intermediate spinal cord electroporated with the indicated constructs relative to the contralateral control side. $*P<0.03$; $**P<0.002$. *Lfng* measurements were taken from the pdl5 and p0 regions for Bhlhb5 mis-expression and the pdl6 region for shRNA knockdown. *Hes5* was measured from the p0-p2 region in both conditions. Plus sign indicates the transfected side of the spinal cord. B5 (red), Bhlhb5; B5 (red and white), Bhlhb5 in the p2/V2 domains; D1/Lf, Dll1 and Lfng; D4, Dll4; $H5^{Low}$, low levels of Hes5; $H5^{High}$, high levels of Hes5; J1, Jag1.

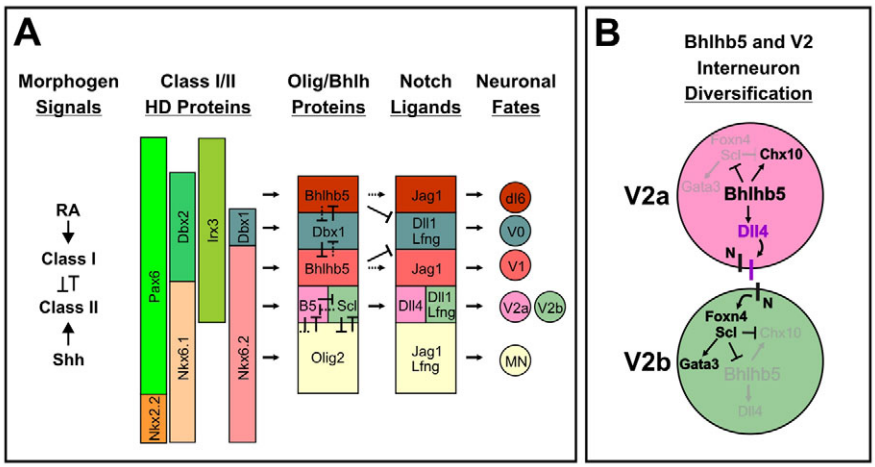

Fig. 8. Consolidation of interneuron fate specification and differentiation through the actions of Bhlhb5 and the Notch signaling pathway. (**A**) Retinoic acid (RA) and Shh signaling activate the expression of Class I and Class II HD proteins in the spinal cord to establish discrete neural progenitor domains. The combinatorial actions of these transcription factors promote Bhlhb5 expression in the intermediate spinal cord. Bhlhb5 helps to refine progenitor domain borders and drives the formation of dI6, V1 and V2 interneurons. Repressive interactions between Bhlhb5 and opposing fate determinants such as Scl subsequently divide V2 neurons into V2a and V2b subclasses. Dashed lines indicate more subtle effects and those primarily supported by gain-of-function evidence. Once progenitor domains are formed, Bhlhb5 regulates the expression of the Notch ligands Dll1 and Jagged1 along with Lfng. (**B**) Within the V2 interneuron lineage, Bhlhb5 promotes V2a differentiation and the expression of Dll4 that engages Notch receptors (N) on neighboring V2 cells to induce Foxn4, Scl and Gata3, and elicit V2b interneuron formation.

Bhlhb5 regulates the pattern of Notch ligand expression, Notch pathway activity and the onset of neuronal differentiation in the spinal cord

The broad reduction of interneuron subtypes in the Bhlhb5 knockdown experiments could be explained by either a change in neuronal differentiation, as occurs with Bhlhb5 mis-expression, or a loss of instructive signals produced by Bhlhb5^{+} cells to promote the formation of specific interneuron subtypes. As the spinal cord develops, Notch ligands and Fringe proteins are expressed in domain-specific patterns that might suppress Notch signaling across domain boundaries but facilitating Notch activation within each domain (Marklund et al., 2010; Ramos et al., 2010). Moreover, within the V2 interneuron lineage, cell-cell communication mediated by Dll4 and Notch receptors enables the emergence of V2a and V2b neurons from a common progenitor pool (Del Barrio et al., 2007; Peng et al., 2007; Rocha et al., 2009). To explore whether the observed non-autonomous effects of Bhlhb5 mis-expression and deficiency might be attributed to changes in the expression of Notch ligands, we mapped the expression of Bhlhb5 relative to Jagged1, Dll1, *Dll4* and the Notch regulator lunatic fringe (*Lfng*) in the unaltered chick spinal cord. We found that Bhlhb5 strikingly overlaps Jagged1 and *Dll4*, and is reciprocal to both Dll1 and *Lfng* (Fig. 7A-D,F).

To examine how Bhlhb5 contributes to this pattern, we examined embryos in which Bhlhb5 had been mis-expressed. Bhlhb5 significantly reduced both Dll1 and *Lfng*, and simultaneously expanded *Dll4* and to a much lesser extent Jagged1 (Fig. 7G-J,Z). These changes in Notch ligand distribution coincided with an elevation of Notch pathway activity in the intermediate spinal cord, reflected by a ~40% increase in the expression of the downstream effector *Hes5* and a corresponding reduction in the number of Ngn2^{+} progenitors and p27^{Kip1+} neurons formed (Fig. 7K,L,X,Z). A similar reduction of Dll1 and *Lfng* and increase in *Dll4* was seen with the combined expression of Bhlhb5 and Ngn2, but not with mis-expression of Ngn alone (see Fig. S7 in the supplementary material), indicating that these effects are specific to Bhlhb5 mis-expression and are not simply a consequence of forced differentiation.

Complementing these mis-expression results, we found that shRNA-mediated knockdown of Bhlhb5 significantly decreased *Dll4* and mildly suppressed Jagged1, and both Dll1 and *Lfng* appeared to expand into the territories where *Dll4* and Jagged1 were normally expressed (Fig. 6C; Fig. 7M-P,R,S,Z). Under these conditions, *Hes5* expression was reduced by ~50%, Ngn2 and NeuroM expanded, and p27^{Kip1+} postmitotic cells began to abnormally appear within the ventricular zone (Fig. 7Q,R,T-V,X,Z). These changes in neuronal differentiation also extended into the Dbx1^{+} p0 progenitor domain (Fig. 7W,Y), suggesting that the observed alterations in Notch ligand and *Lfng* distribution can broadly affect Notch pathway activity and neurogenesis even in regions where Bhlhb5 is not normally expressed. Together, these results provide evidence that, in addition to its cell-autonomous functions in promoting dI6, V1 and V2a interneuron formation, Bhlhb5 regulates the expression of Notch ligands and can thus influence the overall pattern of neuronal differentiation in the spinal cord. Moreover, in directing *Dll4* expression, Bhlhb5 provides an instructive stimulus for V2b neuron development to extend the repertoire of interneuron subtypes generated.

DISCUSSION

In this study, we provide evidence that Bhlhb5 plays a crucial role in the development of the spinal cord, participating first in the formation of progenitor domains and subsequently in the generation of specific classes of interneurons. In addition, Bhlhb5 helps to organize the spatially restricted expression of Notch ligands to regulate the global pattern of neurogenesis and enhance neuronal diversity (Fig. 8). Together with previous studies of the related proteins Olig2 and Olig3 (Mizuguchi et al., 2001; Novitch et al., 2001; Muller et al., 2005), our findings suggest a common function for Olig and Bhlhb transcription factors in translating early patterning information into distinct programs of neuronal

differentiation throughout the spinal cord. In MN progenitors, Shh and RA signaling act through the HD proteins Pax6 and Nkx6 to induce the expression of Olig2, which acts to define the borders of this domain and promote MN differentiation (Mizuguchi et al., 2001; Novitch et al., 2001). Olig3 is activated similarly by Bmp and Wnt signaling in the dorsal spinal cord, where it directs the formation of dI1-dI3 interneurons and suppresses dI4-dI6 fates (Muller et al., 2005; Zechner et al., 2007). Bhlhb5 appears to play an analogous role in the intermediate spinal cord, acting as a downstream effector of RA signaling and Pax6 that helps to establish or maintain progenitor domain borders through its repressive interactions with Dbx1, Olig2 and other targets to promote dI6, V1 and V2a interneuron fates in lieu of others (Fig. 8A).

Our loss-of-function experiments in the chick also suggest a requirement for Bhlhb5 activity within the intermediate spinal cord, as many classes of interneurons were diminished when Bhlhb5 expression was reduced. However, the reduction of Bhlhb5 in our experiments was never complete and, perhaps owing to this limitation, the formation of different classes of interneurons was significantly diminished but not completely lost. The persistence of interneuron development in the knockdown embryos might also reflect redundant or parallel pathways that work in conjunction with Bhlhb5 to direct interneuron differentiation. Indeed, most HD proteins that contribute to the establishment of discrete progenitor domains along the dorsoventral axis were not significantly altered by the loss of Bhlhb5. In addition, we have observed that the closely related gene *Bhlhb4* (*Bhlhe23*) partially overlaps the expression of *Bhlhb5* in the spinal cord, particularly in the mouse, and the combined mis-expression of Bhlhb4 and Ngn2 in the chick can induce ectopic V2a formation much like Bhlhb5 and Ngn2 (K.S. and B.G.N., unpublished data). *Bhlhb5*-mutant mice have recently been described, and although these animals display defects in neuronal differentiation in the retina and cortex (Feng et al., 2006; Joshi et al., 2008) and defects in neuronal survival in some dorsal spinal interneurons (Ross et al., 2010), they do not exhibit the overt motor coordination deficiencies that might be expected if ventral interneuron development were impaired. The generation of *Bhlhb4; Bhlhb5* double knockouts might, thus, be required to reveal the function of these genes in spinal interneuron development. It is further possible that the chick spinal cord is inherently more sensitive to Bhlhb5 loss, as Bhlhb5 appears to be more extensively expressed from the earliest stages of interneuron progenitor formation in chick than in mice.

In addition to its fate-specifying activities, Bhlhb5 appears to help establish the domain-restricted pattern of Notch ligands and Fringe proteins in the spinal cord (Fig. 8A). Bhlhb5 is closely associated with the expression of Jagged1 in both pdI6 and p1 cells, and *Dll4* in V2a interneurons. Neighboring progenitors lacking Bhlhb5 express a different Notch ligand, Dll1, and the Notch regulator *Lfng*. When Bhlhb5 function was reduced, Jagged1 and *Dll4* expression diminished whereas Dll1 and *Lfng* expression expanded, and the opposite outcome was seen with Bhlhb5 mis-expression. It is currently unclear whether these changes reflect a positive effect of Bhlhb5 on Jagged1 and *Dll4* expression or an inhibitory effect on Dll1 and *Lfng*, though the latter seems more likely given that Bhlhb5 appears to carry out most of its functions as a transcriptional repressor. However, our data do not exclude the possibility that the alterations in Notch ligand expression are secondary to the ability of Bhlhb5 to modulate dorsoventral pattern or alter the course of neurogenesis through other pathways. Identification of the genomic targets of Bhlhb5 will be important for distinguishing between these possibilities.

How does the spatial organization of Notch ligands and Fringe proteins contribute to spinal cord development? Recent studies in chick and mouse have shown that manipulating the distribution of Jagged1 and Dll1, either by genetic inactivation or mis-expression, affects predominantly the differentiation behavior of the cells that express a given ligand, though Dll1 signaling can extend more broadly in the absence of Jagged1 (Marklund et al., 2010; Ramos et al., 2010). Hence, progenitors that express Dll1 are sensitive primarily to changes in Dll1 levels and less affected by the changes in Jagged1, and vice versa. These differential sensitivities are attributed to the domain-restricted expression of Lfng and manic fringe, which have the ability to potentiate Dll1 signaling while simultaneously blocking Jagged1 signaling (Hicks et al., 2000; Marklund et al., 2010). Our data indicate that Bhlhb5 plays a crucial role in ensuring that both Notch ligands and Fringe proteins are confined to spatially restricted compartments. This might permit Notch signaling to operate selectively within a given progenitor group without affecting adjacent cells, and thereby enable each progenitor domain to differentiate on a characteristic schedule. Under conditions in which Bhlhb5 activity has been manipulated, this spatial segregation appears to break down, leading to changes in the overall pattern of Notch signaling and neuronal differentiation in the intermediate spinal cord.

The differential expression of Notch ligands and Fringe proteins might act further to preserve or reinforce progenitor domain borders. Notch signaling has an important role in the dorsoventral compartmentalization of the *Drosophila* wing (Major and Irvine, 2005), as well as in the formation of boundary cells in the vertebrate central nervous system (Baek et al., 2006). In this way, the differential expression of Jagged1 and Dll1-Lfng mediated by Bhlhb5 could help prevent the mixing of progenitors to maintain the dorsoventral positions from which neurons and glia emerge over time.

Once neural progenitor domains are established, multiple classes of neurons and glial cells emerge from seemingly homogeneous progenitor populations. Recent studies of V2 interneuron development provide important insights into the molecular mechanisms through which this diversity might be achieved. p2 progenitors produce two functionally distinct classes, excitatory V2a neurons and inhibitory V2b neurons, through cell-cell signaling mediated by Dll4 and Notch receptors (Del Barrio et al., 2007; Peng et al., 2007). Differential Notch activity leads to the assembly of opposing transcription factor complexes that specify the type of V2 interneuron formed: cells that express Bhlhb5 and Dll4 form V2a neurons whereas cells receiving high levels of Notch signaling express Foxn4 and Scl, and consequently adopt the V2b fate (Del Barrio et al., 2007; Peng et al., 2007).

This separation in cell fates could, in principle, occur by a stochastic imbalance in the initially broad expression of Dll4 and Notch receptors within all p2 progenitors, akin to the lateral inhibition model associated with *Drosophila* neuroblast formation (Skeath and Thor, 2003). Alternatively, it could occur in a more deterministic manner, through the concerted actions of Bhlhb5 to establish the pattern of Dll4 expression specifically within V2a neurons in order to elicit the concurrent formation of V2b cells. Our data are most consistent with the latter possibility, considering that Bhlhb5 is detectable in p2 progenitors before the onset of the V2a/V2b subdivision, and that the co-expression of Bhlhb5 alone and in combination with Ngn2 is sufficient to induce V2a

formation and *Dll4* expression throughout the spinal cord. Moreover, knockdown of endogenous Bhlhb5 function reduced both V2a interneuron generation and *Dll4*, and produced a loss rather than gain of V2b neurons as might be expected if the assignments of V2a and V2b fates were independently controlled.

Although Dll4 appears to be essential for V2b generation, Dll4 mis-expression by itself has a very modest ability to stimulate ectopic V2b production and only in cells adjacent to where V2 neurons should normally form (Del Barrio et al., 2007; Peng et al., 2007) (K.S. and B.G.N., unpublished data). These findings are consistent with our observations that the expansion of *Dll4* resulting from Bhlhb5 mis-expression is not sufficient to trigger V2b formation, and suggest that the context in which the Dll4 signal is received is important for cells to adopt the V2b fate. Together, these results lead us to propose that V2 interneuron diversity emerges through a hierarchical relationship between Bhlhb5 and Notch signaling. Bhlhb5 promotes V2a interneuron differentiation leading to *Dll4* expression and the formation of V2b neurons in neighboring cells (Fig. 8B). In this way, Bhlhb5 provides a crucial function in translating the early patterning information bestowed by morphogen signals into local Notch signaling events that both extend neural diversity and ensure that the formation of excitatory and inhibitory neurons is coupled for the coordinated control of motor functions.

Acknowledgements

We thank Alexandria Harrold and Michelle Yang for assistance with shRNA experiments; Alessandra Pierani for *Dbx1*-mutant mouse embryos; David Turner, Stuart Wilson, Tom Jessell, Jonas Muhr, Thomas Müller, Paul Trainor and Yasushi Nakagawa for reagents; and Samantha Butler, James Briscoe, Zachary Gaber and Neil Segil for critical comments on the manuscript and technical advice. K.S. was supported by the University of Michigan Neuroscience Graduate Training Program and the Center for Organogenesis Training Grant (5-T32-HD007505); D.M.M. was supported by the NIH (NS054784 and DC009410); B.G.N. was supported by grants from the Whitehall Foundation (2004-05-90-APL), the March of Dimes Foundation (5-FY2006-281), and the NINDS (NS053976). Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.057281/-/DC1

References

Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-2476.

Batista, M. F., Jacobstein, J. and Lewis, K. E. (2008). Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Dev. Biol.* **322**, 263-275.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.

Briscoe, J. and Novitch, B. G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos. Trans. R. Soc. Lond. B* **363**, 57-70.

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Brunelli, S., Innocenzi, A. and Cossu, G. (2003). Bhlhb5 is expressed in the CNS and sensory organs during mouse embryonic development. *Gene Expr. Patterns* **3**, 755-759.

Burrill, J. D., Moran, L., Goulding, M. D. and Saueressig, H. (1997). PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development* **124**, 4493-4503.

Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.

Das, R. M., Van Hateren, N. J., Howell, G. R., Farrell, E. R., Bangs, F. K., Porteous, V. C., Manning, E. M., McGrew, M. J., Ohyama, K., Sacco, M. A. et al. (2006). A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev. Biol.* **294**, 554-563.

Dasen, J. S., De Camilli, A., Wang, B., Tucker, P. W. and Jessell, T. M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* **134**, 304-316.

Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D. I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., Xiang, M., Rowitch, D. et al. (2007). A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* **134**, 3427-3436.

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C. J., Gronostajski, R. M. and Anderson, D. J. (2006). The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**, 953-968.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Feng, L., Xie, X., Joshi, P. S., Yang, Z., Shibasaki, K., Chow, R. L. and Gan, L. (2006). Requirement for Bhlhb5 in the specification of amacrine and cone bipolar subtypes in mouse retina. *Development* **133**, 4815-4825.

Goulding, M. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. *Nat. Rev. Neurosci.* **10**, 507-518.

Goulding, M. and Pfaff, S. L. (2005). Development of circuits that generate simple rhythmic behaviors in vertebrates. *Curr. Opin. Neurobiol.* **15**, 14-20.

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-549.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* **2**, 515-520.

Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q. and Anderson, D. J. (2008). Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* **133**, 510-522.

Joshi, P. S., Molyneaux, B. J., Feng, L., Xie, X., Macklis, J. D. and Gan, L. (2008). Bhlhb5 regulates the postmitotic acquisition of area identities in layers II-V of the developing neocortex. *Neuron* **60**, 258-272.

Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.* **29**, 279-306.

Kimura, Y., Satou, C. and Higashijima, S. (2008). V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. *Development* **135**, 3001-3005.

Li, S., Misra, K., Matise, M. P. and Xiang, M. (2005). Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. *Proc. Natl. Acad. Sci. USA* **102**, 10688-10693.

Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell. Neurosci.* **8**, 14-27.

Liu, B., Liu, Z., Chen, T., Li, H., Qiang, B., Yuan, J., Peng, X. and Qiu, M. (2007). Selective expression of Bhlhb5 in subsets of early-born interneurons and late-born association neurons in the spinal cord. *Dev. Dyn.* **236**, 829-835.

Major, R. J. and Irvine, K. D. (2005). Influence of Notch on dorsoventral compartmentalization and actin organization in the Drosophila wing, *Development* **132**, 3823-3833.

Marklund, U., Hansson, E. M., Sundström, E., Angelis, M. H. D., Przemeck, G. K. H., Lendahl, U., Muhr, J. and Ericson, J. (2010). Domain-specific control of neurogenesis achieved through patterned regulation of Notch ligand expression. *Development* **137**, 437-445.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K. and Nakafuku, M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**, 757-771.

Mukouyama, Y. S., Deneen, B., Lukaszewicz, A., Novitch, B. G., Wichterle, H., Jessell, T. M. and Anderson, D. J. (2006). Olig2+ neuroepithelial motoneuron progenitors are not multipotent stem cells in vivo. *Proc. Natl. Acad. Sci. USA* **103**, 1551-1556.

Muller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-562.

Muller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M. and Birchmeier, C. (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev.* **19**, 733-743.

Muroyama, Y., Fujiwara, Y., Orkin, S. H. and Rowitch, D. H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature* **438**, 360-363.
Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.
Novitch, B. G., Chen, A. I. and Jessell, T. M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31**, 773-789.
Novitch, B. G., Wichterle, H., Jessell, T. M. and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40**, 81-95.
Osumi, N., Hirota, A., Ohuchi, H., Nakafuku, M., Iimura, T., Kuratani, S., Fujiwara, M., Noji, S. and Eto, K. (1997). Pax-6 is involved in the specification of hindbrain motor neuron subtype. *Development* **124**, 2961-2972.
Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L. and Sharma, K. (2007). Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**, 813-827.
Peyton, M., Stellrecht, C. M., Naya, F. J., Huang, H. P., Samora, P. J. and Tsai, M. J. (1996). BETA3, a novel helix-loop-helix protein, can act as a negative regulator of BETA2 and MyoD-responsive genes. *Mol. Cell. Biol.* **16**, 626-633.
Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.
Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**, 367-384.
Ramos, C., Rocha, S., Gaspar, C. and Henrique, D. (2010). Two Notch ligands, Dll1 and Jag1, are differently restricted in their range of action to control neurogenesis in the mammalian spinal cord. *PLoS ONE* **5**, e15515.
Rocha, S. F., Lopes, S. S., Gossler, A. and Henrique, D. (2009). Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. *Dev. Biol.* **328**, 54-65.
Ross, S. E., Mardinly, A. R., McCord, A. E., Zurawski, J., Cohen, S., Jung, C., Hu, L., Mok, S. I., Shah, A., Savner, E. M. et al. (2010). Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. *Neuron* **65**, 886-898.
Rousso, D. L., Gaber, Z. B., Wellik, D., Morrisey, E. E. and Novitch, B. G. (2008). Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. *Neuron* **59**, 226-240.
Rowitch, D. H., Lu, Q. R., Kessaris, N. and Richardson, W. D. (2002). An 'oligarchy' rules neural development. *Trends Neurosci.* **25**, 417-422.
Ruzinova, M. B. and Benezra, R. (2003). Id proteins in development, cell cycle and cancer. *Trends Cell Biol.* **13**, 410-418.
Sandberg, M., Kallstrom, M. and Muhr, J. (2005). Sox21 promotes the progression of vertebrate neurogenesis. *Nat. Neurosci.* **8**, 995-1001.
Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.
Sockanathan, S. and Jessell, T. M. (1998). Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* **94**, 503-514.
Stepien, A. E. and Arber, S. (2008). Probing the locomotor conundrum: descending the 'V' interneuron ladder. *Neuron* **60**, 1-4.
Vue, T. Y., Aaker, J., Taniguchi, A., Kazemzadeh, C., Skidmore, J. M., Martin, D. M., Martin, J. F., Treier, M. and Nakagawa, Y. (2007). Characterization of progenitor domains in the developing mouse thalamus. *J. Comp. Neurol.* **505**, 73-91.
Wichterle, H., Lieberam, I., Porter, J. A. and Jessell, T. M. (2002). Directed differentiation of embryonic stem cells into motor neurons. *Cell* **110**, 385-397.
Xu, Z. P., Dutra, A., Stellrecht, C. M., Wu, C., Piatigorsky, J. and Saunders, G. F. (2002). Functional and structural characterization of the human gene BHLHB5, encoding a basic helix-loop-helix transcription factor. *Genomics* **80**, 311-318.
Yeo, S. Y. and Chitnis, A. B. (2007). Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. *Proc. Natl. Acad. Sci. USA* **104**, 5913-5918.
Yuan, C., Zins, E. J., Clark, A. F. and Huang, A. J. (2007). Suppression of keratoepithelin and myocilin by small interfering RNAs (siRNA) in vitro. *Mol. Vis.* **13**, 2083-2095.
Zechner, D., Muller, T., Wende, H., Walther, I., Taketo, M. M., Crenshaw, E. B., 3rd, Treier, M., Birchmeier, W. and Birchmeier, C. (2007). Bmp and Wnt/beta-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons. *Dev. Biol.* **303**, 181-190.
Zhou, Q., Choi, G. and Anderson, D. J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. *Neuron* **31**, 791-807.

Table S1. Primers used to generate in situ probes

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Scl	GAGGAATTCGGACGCTCGGTGTTTAGGTA	GAGATTAACCCTCACTAAAGGGACTTTTGCTGAGGGCATTTTC
Dll4	CTTCTGGGCCATGTGAGAAT	GAGTATTTAGGTGACACTATAGGTTCCATCCTCCACCTGAGA
Lfng	CCTTGGTCAGGGTCACAGTT	GAGAATTAACCCTCACTAAAGGGAGAGGGGCACCTGTGTTTTTA
Hes5 isoform 2 (*Hes5-2*)	TTCCAAAGGAAAAACCAACG	GAGATTAACCCTCACTAAAGGGAACGTCTGTAGCGACCCTTTG

Underlined regions indicate a SP6 or T3 RNA polymerase site embedded in the reverse primers.