

# Control of Roof Plate Development and Signaling by *Lmx1b* in the Caudal Vertebrate CNS

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Numerous studies have identified the roof plate as an important signaling center controlling dorsal interneuron specification and differentiation in the developing spinal cord. Currently, the molecular pathways of roof plate formation and function are poorly understood. We determined that the LIM-homeodomain transcription factor *Lmx1b* is sufficient to induce functional roof plate in the early chick developing spinal cord. In the chick, *Lmx1b* acts upstream of *Lmx1a* in the roof plate developmental program. Once the roof plate forms, we show that Bmp and Wnt signaling are the major components of *Lmx1a/b*-dependent roof plate dorsal patterning activity. The roof plate function of *Lmx1b* is not conserved across vertebrates because *Lmx1b* is not expressed in mouse roof plate progenitors. Instead, mouse caudal CNS roof plate formation relies entirely on *Lmx1a*. *Lmx1b* can, however, partially rescue roof plate development in *dreher* (*Lmx1a*<sup>-/-</sup>) mice, indicating that *Lmx1b* has some functional redundancy to *Lmx1a*. Furthermore, we demonstrate that the roof plate-inducing activity of *Lmx1b* can be suppressed by *Mash1* (*Cash1*), which is normally expressed in intermediate neural tube in both chick and mouse. Our data identify *Lmx1b* as a key regulator of spinal cord roof plate induction and function.

**Key words:** *Lmx1b*; roof plate; *Lmx1a*; *dreher*; Bmp; Wnt; developing spinal cord

## Introduction

The roof plate of the developing CNS is a transient non-neural dorsal midline structure that controls the specification, differentiation, and axonal trajectories of the neurons at the dorsal neural tube (Lee and Jessell, 1999; Butler and Dodd, 2003). In caudal CNS, the roof plate originates from mitotically active progenitors of the neural folds in response to Bmp signaling from the adjacent epidermal ectoderm (Liem et al., 1997; Lee and Jessell, 1999). As development proceeds, roof plate progenitors exit the cell cycle, acquire typical roof plate morphology, and initiate expression of differentiated roof plate markers, including *MafB*. In addition, the roof plate secretes peptides of the Bmp and Wnt families, which have been proposed to play major roles in the specification of adjacent dorsal interneurons both *in vitro* and *in vivo* (Liem et al., 1997; Lee and Jessell, 1999; Muroyama et al., 2002; Timmer et al., 2002; Helms and Johnson, 2003). Although numerous studies have firmly established the importance of the roof plate as a signaling center controlling dorsal CNS patterning, little is understood regarding the molecular pathways driving roof plate induction or function.

We established previously that the LIM-homeodomain protein *Lmx1a* is required for roof plate formation. *Lmx1a* is expressed in the neural folds and in the developing roof plate (Millonig et al., 2000; Failli et al., 2002). Loss of *Lmx1a* function in *dreher* mutant mice leads to the failure of roof plate formation in the caudal CNS and subsequent disruption of dorsal neural patterning (Millonig et al., 2000). *Lmx1a* is a member of a large family of LIM-homeobox-encoding genes. Within this large, diverse family, *Lmx1a* is most highly related to *Lmx1b*, a protein with 61% overall amino acid identity (Hobert and Westphal, 2000).

*Lmx1b* is widely expressed during embryogenesis, and its function is required for normal limb, kidney, eye, and skull development (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998; Dreyer et al., 1998). In the CNS, *Lmx1b* is expressed in the isthmus, developing serotonergic and dopaminergic neurons of the hindbrain and midbrain, and a subset of dorsal interneurons of the spinal cord. *Lmx1b* is also expressed in the roof plate and floor plate of the neural tube (Yuan and Schoenwolf, 1999; Adams et al., 2000; Smidt et al., 2000; Gross et al., 2002; Matsunaga et al., 2002; Muller et al., 2002; Ding et al., 2003). Although the role of *Lmx1b* in the isthmus (Adams et al., 2000; Matsunaga et al., 2002) and serotonergic and dopaminergic neurons (Smidt et al., 2000; Ding et al., 2003) is clearly established, its function in dorsal spinal cord development is unclear.

Using chick *in ovo* electroporation technology in conjunction with mutant mouse analysis, we determined that roof plate induction is the primary activity of *Lmx1b* in the early developing dorsal spinal cord. *Lmx1b* expression in the developing roof plate is activated by Bmp expression, and its roof plate-inducing activity can be inhibited by *Mash1* (*Cash1*), normally expressed in intermediate neural tube. Strikingly, *Lmx1b* is not expressed in

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the roof plate of the mouse caudal CNS. Mouse spinal cord roof plate development is entirely dependent on *Lmx1a*. *Lmx1b* acts upstream of *Lmx1a* in the chick roof plate differentiation program and can partially rescue roof plate development in the *dreher* mouse. Using both gain-of-function and loss-of-function approaches, we identified Bmp signaling as the major component of *Lmx1a/b*-dependent roof plate signaling in chick developing spinal cord. *Lmx1a/b* also activates dorsal midline Wnt1 expression. The role of *Lmx1a/b*-dependent Wnt1 expression, however, is limited, regulating the numbers of only the most dorsal neuronal population, the dI1 interneurons.

## Materials and Methods

**Embryos.** Fertilized White Leghorn eggs were incubated and staged according to Hamburger and Hamilton (1951). *dreher* (*Lmx1a*<sup>-/-</sup>) and *Lmx1b* mutant mice were maintained and genotyped as described previously (Chen et al., 1998; Millonig et al., 2000).

**Chick embryo manipulations.** *In ovo* electroporation was used to express proteins of interest in chick developing spinal cord as described previously (Megason and McMahon, 2002). Full-length mouse *Lmx1b* was cloned upstream of internal ribosomal entry site followed by enhanced green fluorescent protein (IRES-EGFP) in the pCIG expression vector (Megason and McMahon, 2002). Chick *Lmx1b* was expressed using chick *Lmx1b*/RCAS(A) virus (Riddle et al., 1995). Mouse *Lmx1a*, *Bmp4*, *Wnt1*, dominant-negative (*dn*) *Wnt1*, chick *Noggin*, and *Xenopus Follistatin* were expressed from pCIG-*Lmx1a* (Chizhikov and Millen, 2004), pMiwII-BMP4 (Kishimoto et al., 2002), pCIG-Wnt1 (Megason and McMahon, 2002), pC1-neo-dnWnt1 (Garcia-Castro et al., 2002), pMT-Noggin, and pCMV-Follistatin (Liem et al., 2000), respectively. In all experiments, an appropriate empty vector was used as a control. In cell cycle experiments, 150  $\mu$ l of different concentrations of olomoucine or aphidicolin (Sigma, St. Louis, MO) were applied on top of embryos *in ovo* 5 hr after electroporation (h.a.e.), corresponding to the approximate initial expression of the exogenous *dnWnt1*. Chick intermediate explants from electroporated and control neural plates were isolated according to Liem et al. (1995) and cultured in serum-free medium as described previously (Garcia-Castro et al., 2002).

**Mouse embryo manipulations.** Embryonic day 9.25 (E9.25) whole *dreher* embryos or their subdissected neural tubes (Lee et al., 1998) were embedded into collagen and electroporated as described previously (Akamatsu et al., 1999). They were then cultured for 24 hr using previously published conditions (Lee et al., 1998). All cultured embryos and some neural tube explants were processed for immunohistochemistry. Some neural tube explants were used for RNA isolation and reverse transcription (RT)-PCR analysis.

**Immunohistochemistry, in situ hybridization, bromodeoxyuridine, and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.** Immunohistochemistry was performed on frozen sections as described previously (Helms and Johnson, 1998). The following primary antibodies were used: anti-MafB (Pouponnot et al. 1995), anti-LH2A/B (Lee et al., 1998), anti-Math1 (Helms and Johnson, 1998), anti-Lbx1 (Muller et al., 2002), anti-*Lmx1a* (M. German, unpublished observation; a gift from M. German, University of California San Francisco, San Francisco, CA), anti-Brn3a (Fedtsova and Turner, 1997), anti-*Lmx1b*, anti-Islet1 (51.4H9), anti-Lim1/2 (4F2), anti-Msx1/2 (4G1), anti-Pax7, and anti-Pax6 [all obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), developed under the auspices of the National Institute of Child Health and Human Development]. Secondary species-appropriate antibodies with Texas Red or FITC conjugates were obtained from Jackson ImmunoResearch (West Grove, PA). *In situ* hybridization was performed as described previously (Timmer et al., 2002) using digoxigenin-labeled riboprobes to chick *Gdf7*, *Bmp4*, *Wnt1*, *Cash1*, and mouse *Lmx1b* provided by T. Jessell (Columbia University, New York, NY) (*Gdf7*), P. Brickell (Institute of Child Health, London, UK) (*Bmp4*), A. McMahon (Harvard University, Cambridge, MA) (*Wnt1*), J. Johnson (University of Texas Southwestern Medical Center, Dallas, TX) and D. Anderson (California Institute of Technology, Pasadena, CA) (*Cash1*), and R. Johnson (University of Texas, M.D.

Anderson Cancer Center, Houston, TX) (*Lmx1b*). Bromodeoxyuridine (BrdU) and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling were performed as described previously (Megason and McMahon, 2002). Sections were digitally photographed with an AxioCam on a Zeiss (Oberkochen, Germany) AxioPlan 2 microscope and processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

**RT-PCR.** Each explant was collected into 200  $\mu$ l of extraction buffer, and RNA was extracted using PicoPure RNA extraction kit (Arcturus, Mountain View, CA). Total RNA was reverse transcribed by use of random primers. cDNA was amplified by PCR for 30 cycles. Primer pairs to amplify mouse transcripts were 5'-TCACTGCACGTGGACTTTAAG-GAG-3' and 5'-TGATGGGACTGAGCCTTGCG-3' for *Gdf7*, and 5'-ACTGCCGTCGCCGTCGCCATTCACTA-3' and 5'-CACCACCTGTGTCATACTCATCCAG-3' for *Bmp4*. Amplification of a fragment of GAPDH was also performed as a control with primers 5'-TGACGTGCCGCTGGAGAAA-3' and 5'-GGTCCACCACCCTGTGCTGTA-3'. PCR products were fractionated in 2% agarose gels and visualized by staining with ethidium bromide.

**Cell count and statistical analysis.** All sections were taken from the region between forelimbs and hindlimbs. Only sections with sufficient levels of GFP expression (10–15 representative sections per embryo) were used for analysis. All results were replicated in at least four embryos or explants. For quantification of roof plate cells, the total number of positive cells was counted on each section. For quantification of dorsal interneurons, only positive cells on the electroporated side were counted on each section. These numbers were compared with numbers of cells of interest in GFP-electroporated control embryos and not to the numbers of on the non-electroporated slides of the experimental embryos, because we sometimes observed that *Lmx1b*-induced roof plate non-autonomously affected not only the electroporated but also the control side of the neural tube. All quantitative data are expressed as the mean  $\pm$  SEM. Statistical significance was determined by two-tailed *t* test. \* indicates *p* < 0.01 in all figures.

## Results

### Roof plate induction by *Lmx1b* in chick developing dorsal spinal cord

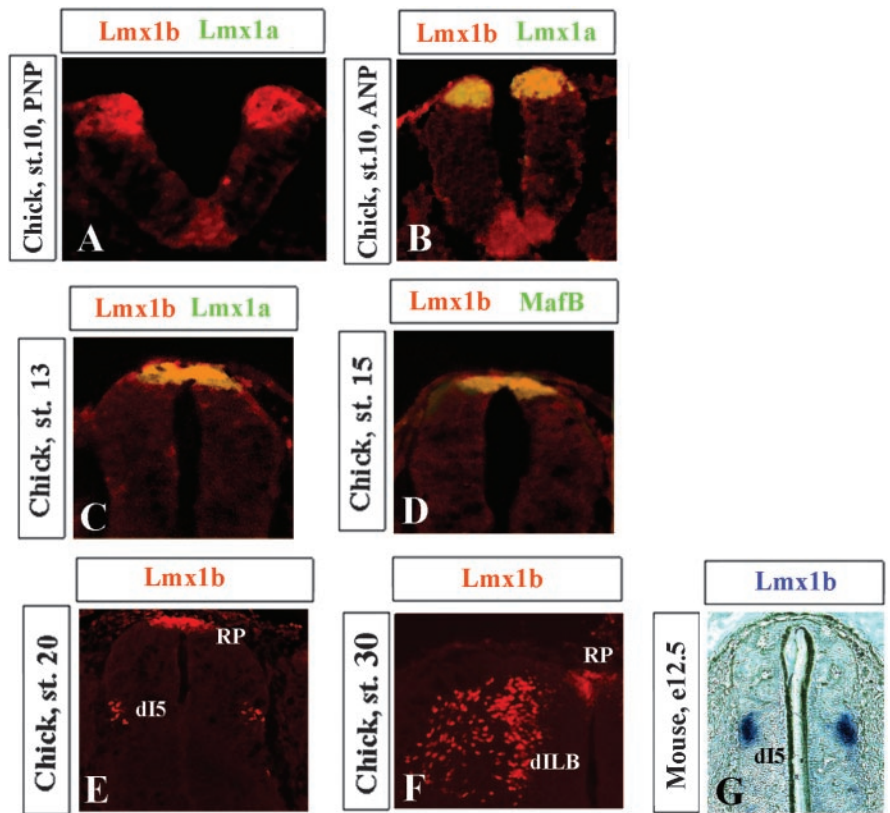
In the chick developing dorsal spinal cord, *Lmx1b* is expressed in the dorsal midline, and dorsal dI5 and dILB interneurons (Fig. 1A–F) (Yuan and Schoenwolf, 1999; Gross et al., 2002; Muller et al., 2002). In the chick caudal CNS, we first detected *Lmx1b* expression at the beginning of neurulation, specifically in the elevating and converging neural folds (Fig. 1A). Just before neural tube closure, expression of the roof plate marker *Lmx1a* was then initiated. In both the neural folds and dorsal midline of the formed neural tube at later stages, the *Lmx1b* expression completely overlapped with the *Lmx1a* expression domain (as detected by double immunolabeling) (Fig. 1B, C). *Lmx1b* expression also completely overlapped with MafB, a specific marker of differentiated roof plate that is first expressed at stage 15 (Fig. 1D). By electroporating stage 10 chick neural plates with a *Bmp4* expression vector, we showed that, similar to other roof plate markers (MafB, *Bmp4*, *Wnt1*), roof plate *Lmx1b* expression is positively regulated by Bmp signaling (data not shown) (Liem et al., 1997; Liu et al., 2004). Strikingly, *Lmx1b* expression was not detected in the neural folds or in the dorsal midline of the developing spinal cord in the mouse at any stage investigated (Fig. 1G), although, in the mouse, *Lmx1b* is clearly expressed in the dI5 and dILB dorsal interneurons (Fig. 1G and data not shown) (Gross et al., 2002; Muller et al., 2002).

Because *Lmx1b* is highly expressed in roof plate progenitors and differentiated roof plate cells in the chick developing spinal cord, we investigated whether *Lmx1b* could induce roof plate fate. We overexpressed chick and mouse *Lmx1b* in caudal neural plate shortly before neural tube closure using *in ovo* electroporation of

chick embryos at stage 10, the time of initiation of endogenous *Lmx1b* expression in the neural folds. In all experiments described below, overexpression of chick and mouse *Lmx1b* resulted in identical phenotypes (all figures show overexpression of chick *Lmx1b*).

Roof plate marker analysis of embryos electroporated with *Lmx1b* revealed an approximate fourfold increase in the number of dorsal MafB-positive cells compared with embryos electroporated with EGFP alone (Fig. 2*A–E*). In addition, the expression domains of secreted roof plate markers *Gdf7*, *Bmp4*, and *Wnt1* were also ectopically expanded in embryos electroporated with *Lmx1b* (10 of 14 embryos) but not EGFP alone ( $n = 6$  embryos) (Fig. 2*F–I* and data not shown). The ectopic expression of all roof plate markers was limited to the most dorsal region of the neural tube, despite widespread electroporation of exogenous *Lmx1b* ( $n = 14$ ) (Fig. 2*A, B* and data not shown), suggesting a regionally restricted competence to produce roof plate cells in response to *Lmx1b* activity. An alternative hypothesis is that *Lmx1b* can only expand already existing roof plate and is not sufficient to induce it *de novo*. To distinguish these two possibilities, *in vitro* explant experiments were performed. Control stage 10 intermediate neural tube naive explants ( $n = 7$ ) (Liem et al., 1997) or those electroporated with EGFP alone ( $n = 5$ ) failed to generate any roof plate cells, as assessed by Pax7/MafB double staining (Fig. 2*L–N*). After 30 hr in culture, however, these control explants initiated coexpression of endogenous *Lmx1b* and *Lbx1*, the generic marker of dI4–dI6 interneurons (data not shown). Because in developing spinal cord this marker combination is unique to dI5 dorsal interneurons (Gross et al., 2002; Muller et al., 2002), we conclude that some explant cells adopted dI5 interneuron fate in the absence of dorsaling or ventralizing signals. In contrast, intermediate explants electroporated with *Lmx1b* produced numerous roof plate cells ( $n = 4$  explants) (Fig. 2*J, K, N*), indicating that *Lmx1b* can induce roof plate *de novo*. No expression of *Lbx1* was detected in these explants at any time investigated (data not shown). Thus, *Lmx1b* can induce roof plate *de novo* in stage 10 naive intermediate neural plate explants and can either directly or indirectly override the dI5 cell fate when it is expressed at this early stage.

Because endogenous roof plate consists of non-dividing differentiated cells, we investigated whether *Lmx1b* expression was sufficient to withdraw neural progenitors from the cell cycle. Surprisingly, many cells expressing exogenous *Lmx1b* were still BrdU positive as late as 48 h.a.e., and no differences in numbers of BrdU-incorporating cells were detected between *Lmx1b*-electroporated and control sides of the neural tubes (supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org)). Additionally, no changes in BrdU incorporation were detected when *Lmx1b* was overexpressed in subdissected E9.25 mouse neural tubes *in vitro* (data not shown). Thus, although *Lmx1b* can induce expression of all roof plate markers investigated, we found no evidence that *Lmx1b* alters the cell cycle of electroporated cells.



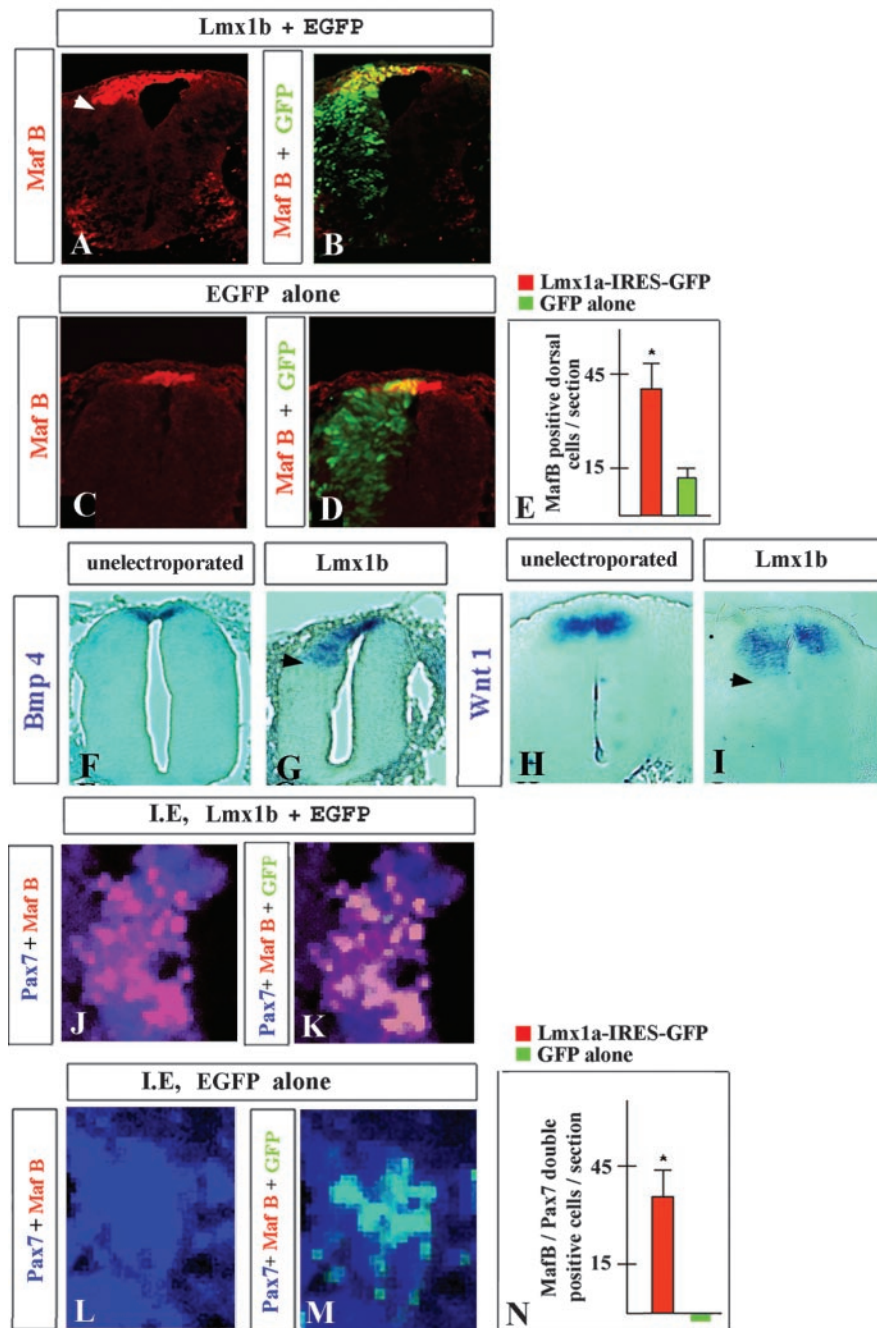
**Figure 1.** Expression of *Lmx1b* in chick and mouse developing spinal cord. Immunofluorescence labeling of *Lmx1b* (red) alone (*E, F*) or together with *Lmx1a* (green) (*A–C*), and *MafB* (green) (*D*) in chick developing spinal cord at the stages (st.) indicated. PNP, Posterior neural plate; ANP, anterior neural plate. Cells that coexpress two markers appear yellow. Expression of *Lmx1b* (blue) detected by *in situ* hybridization in E12.5 mouse developing spinal cord. RP, Roof plate.

### Functional activity of *Lmx1b*-induced roof plate

The primary function of roof plate in developing spinal cord is to pattern adjacent dorsal tissue (Lee and Jessell, 1999). To investigate whether the ectopic roof plate induced by exogenous *Lmx1b* *in vivo* was functional, we first examined the expression of Pax and Msx family members as early indicators of regional identity in developing spinal cord (Liem et al., 1995; Ericson et al., 1997; Timmer et al., 2002). Additionally, we assessed the patterning of dI1, dI2, dI3, and dI4–dI6 dorsal spinal cord interneurons (Fig. 3*Q*). These were identified by their expression of the LIM-homeodomain proteins LH2A/B, *Lim1/2*, *Islet1*, and *Lbx1*, respectively (Gross et al., 2002; Muller et al., 2002) (Fig. 3). To avoid the complications of any potential cell-autonomous effects of *Lmx1b* on dorsal interneuron cell fate, we limited our analysis to sections in which exogenous *Lmx1b* expression was restricted to only the most dorsal regions of the neural tube (as detected by GFP fluorescence and confirmed by anti-GFP antibody staining) (Fig. 3*B, D, F, I, L, O* and data not shown).

In unelectroporated embryos, Pax7 and *Msx1/2* expression was restricted to dorsal cells of the neural tube (Liem et al., 1995; Timmer et al., 2002). Pax6 was expressed at high levels in the intermediate regions of the neural tube and at reduced levels dorsally (Ericson et al., 1997; Timmer et al., 2002). In the neural tubes of *Lmx1b*-electroporated embryos, however, the expression domains of Pax7 and *Msx1/2* were significantly extended ventrally (Fig. 3*A, B* and data not shown). The Pax6 expression domain was reduced and also shifted ventrally (Fig. 3*C, D*). There was an approximate fourfold increase in numbers of dI1 inter-





**Figure 2.** Lmx1b induces roof plate in chick developing spinal cord. Expression of MafB (A–D) (red, detected by immunofluorescence), *Bmp4* (F, G), and *Wnt1* (H, I) (both blue, detected by *in situ* hybridization) in neural tubes of unelectroporated embryos (F, H) and embryos electroporated with either *Lmx1b*–EGFP (A, B, G, I) or EGFP alone (C, D). All pictures are from stage 24 embryos, 48 h.a.e. Arrowheads point to the ventral boundary of ectopic domains of expression of roof plate markers induced by exogenous *Lmx1b*. E, Quantitative analysis of dorsal MafB-positive cells in embryos electroporated with *Lmx1b* and EGFP alone. J–M, Expression of MafB (red) and Pax7 (blue) (J, L) or MafB and Pax7 together with GFP (green) (K, M) and quantitative analysis of roof plate cells (N) in chick intermediate neural plate explants (I.E.) electroporated with *Lmx1b*–EGFP (J, K) or EGFP alone (L, M).

neurons in samples expressing exogenous *Lmx1b*. Additionally, there was a marked shift of these interneurons to more ventral regions of the neural tube (eight of nine embryos), areas normally populated by dI2 and dI3 interneurons (Fig. 3E–G). No dI2, dI3, or dI4–dI6 interneurons were detected in neural tubes with high levels of exogenous *Lmx1b* expression in the dorsal midline region (Fig. 3H–P). We conclude that the ectopic roof plate in-

duced by exogenous *Lmx1b* is functional because it repatterns the regional identity of the developing neural tube (summarized in Fig. 3Q), non-autonomously altering dorsal neuronal specification.

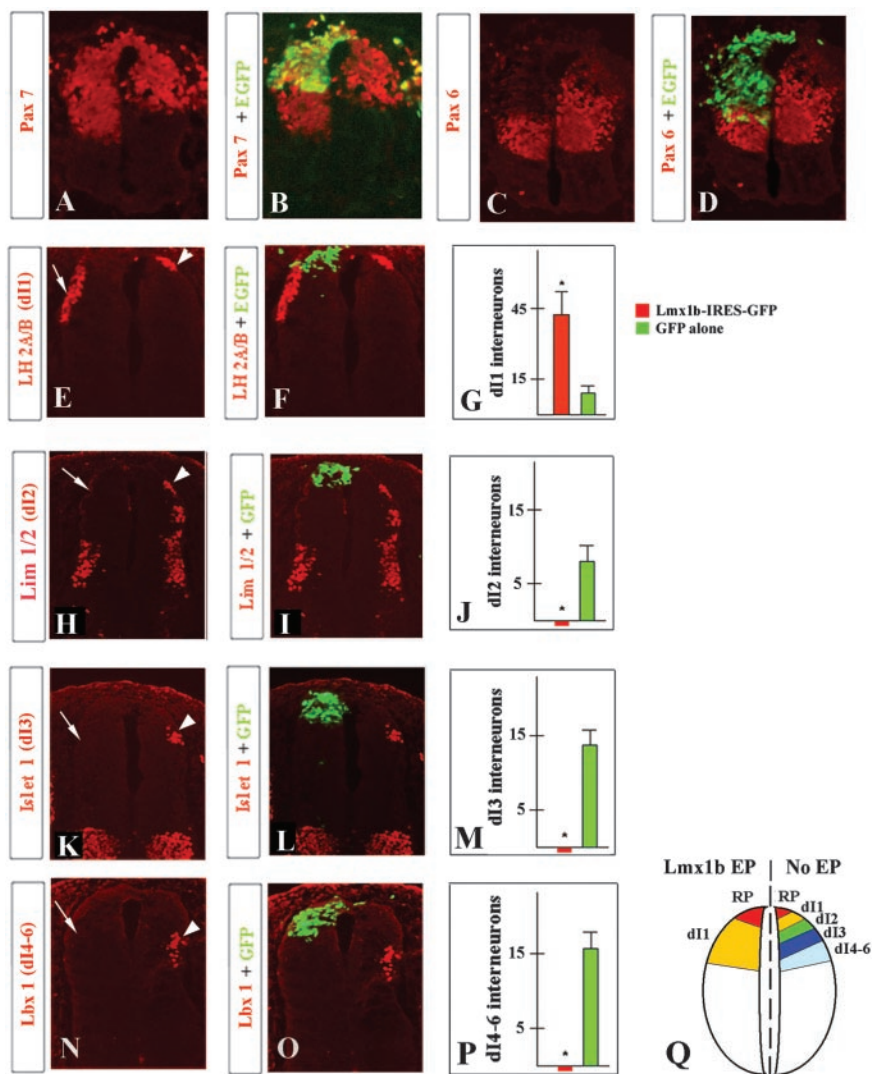
### Lmx1b acts upstream of Lmx1a in the chick roof plate differentiation program and can partially rescue roof plate development in *dreher* mouse embryos

*Lmx1b* can induce *Lmx1a* expression when ectopically expressed in both developing spinal cord *in vivo* (five of seven embryos) (Fig. 4A–C) and in chick naive intermediate neural plate explants *in vitro* ( $40 \pm 7$  *Lmx1a*-positive cells per section; five of six explants) (data not shown). In contrast, *Lmx1a* could not induce *Lmx1b* expression either *in vivo* ( $n = 5$  embryos) (Fig. 4D–F) or *in vitro* ( $n = 4$  explants) (data not shown), indicating that *Lmx1b* acts upstream of *Lmx1a* in the roof plate differentiation program. We determined previously that *Lmx1a* is sufficient to induce the entire roof plate differentiation program in chick developing spinal cord (our published observations). Thus, the induction of the roof plate by *Lmx1b* may be explained by the activation of *Lmx1a* expression. To determine whether the *Lmx1b* roof plate-inducing activity relies entirely on *Lmx1a*, we performed mouse electroporation experiments. We electroporated E9.25 *dreher* (*Lmx1a*<sup>−/−</sup>) embryos or their subdivided neural tubes with either chick or mouse *Lmx1b*-expressing or control vectors, cultured them *in vitro* for 24 hr, and then stained them with antibodies for gene expression analysis by RT-PCR. As expected, unelectroporated *dreher* embryos or those electroporated with EGFP did not express MafB (Fig. 4H, J, K), *Gdf7*, or *Bmp4*, as detected by RT-PCR (Fig. 4L). In contrast, *dreher* embryos electroporated with either chick or mouse *Lmx1b* had numerous MafB-positive cells in the dorsal midline domain of their developing spinal cord (Fig. 4I–K). Strong *Gdf7* and *Bmp4* expression was detected in their neural tubes (Fig. 4L). These data indicate that, although *Lmx1b* is not expressed in mouse caudal roof plate, both chick and mouse *Lmx1b* can at least partially rescue roof plate development in *dreher* mouse embryos. These assays, however, cannot distinguish between the possibility that *Lmx1b* has similar functional capacity to *Lmx1a* or whether *Lmx1b* can induce roof plate via an independent pathway.

**The molecular nature of Lmx1-dependent roof plate signaling**  
Bmps and Wnts have been implicated in the development of several classes of dorsal interneurons (Liem et al., 1997; Murayama et al., 2002; Timmer et al., 2002; Liu et al., 2004), al-

though many details of their specific roles remain unclear. Because *Lmx1a/b* can induce functional roof plate, manipulation of *Lmx1a/b* expression provides an opportunity to investigate the molecular nature of *Lmx1*-dependent roof plate signaling. We first established that overexpression of *Bmp4* in stage 15 chick caudal neural tubes resulted in a phenotype that was indistinguishable from the *Lmx1b* overexpression phenotype (compare supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org), with Fig. 3), suggesting that *Bmp* signaling likely represents a major component of *Lmx1b*-dependent roof plate signaling. In contrast, ectopic expression of *Wnt1* had a very limited effect on dorsal spinal cord development, affecting only dI1 interneuron development (supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org)). *Wnt1* overexpression led to a threefold increase in dI1 population (supplemental Fig. 2H, J, available at [www.jneurosci.org](http://www.jneurosci.org)). However, unlike the phenotype caused by *Lmx1b* overexpression, these dI1 interneurons were still located in their endogenous domain and not significantly expanded ventrally (supplemental Fig. 2H, available at [www.jneurosci.org](http://www.jneurosci.org)). Although previous studies have found that *Wnt1* has a mitogenic effect (Megason and McMahon, 2002), the observed increase in dI1 numbers in *Wnt1*-electroporated embryos cannot be completely explained by the mitogenic activity of *Wnt1*, because the numbers of other dorsal interneurons (such as dI2, dI3, and dI4–dI6) in *Wnt1*-electroporated neural tubes were only moderately increased (supplemental Fig. 2I, J, available at [www.jneurosci.org](http://www.jneurosci.org), and data not shown).

Next, we used an inhibitor approach to directly test the role of *Bmp* and *Wnt* signaling as components of *Lmx1a/b*-dependent roof plate signaling. Coelectroporation of *Lmx1b* or *Lmx1a* with either the *Bmp* inhibitors *noggin* and *folistatin* (Liem et al., 1997) or the *Wnt* inhibitor *dnWnt1* (Garcia-Castro et al., 2002) did not affect the ability of *Lmx1* genes to induce *MafB*-positive cells both *in vivo* and in intermediate neural plate explants *in vitro* (data not shown). These data indicate that the *Bmps* and *Wnts* are not required downstream of *Lmx1a/b* to form roof plate. In contrast, however, *Bmp* signaling from the *Lmx1a/b*-induced roof plate is required for roof plate function, because coelectroporation of *Lmx1a/b* together with *noggin* and *folistatin* blocked the ability of *Lmx1* genes to cause ventral expansion of *Pax7* and *Msx1/2* expression domains. Instead, these electroporations caused a decrease in *Pax7* and *Msx1/2* expression in dorsal neural tube and a dorsal shift in the *Pax6* expression domain (Fig. 5A, B and data not shown). Coelectroporation of *Lmx1a/b* with *noggin* and *folistatin* also blocked the *Lmx1*-dependent loss of dI4–dI6 interneurons and, instead, expanded their domain dorsally (Fig. 5D, F and data not shown). Additionally, the *Bmp* inhibitors prevented the ventral extension of dI1 interneurons (Fig. 5C) that was always observed when *Lmx1b* or *Lmx1a* was overexpressed alone. Importantly, however, a



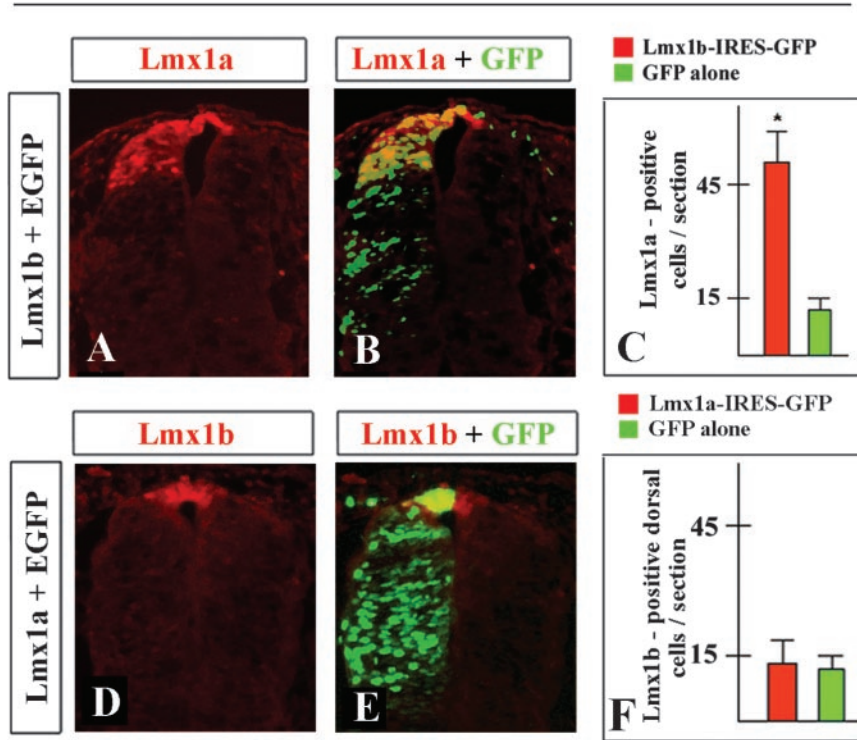
**Figure 3.** Effects of ectopic *Lmx1b*-generated roof plate on dorsal and intermediate spinal cord specification. Expression of *Pax7* (A, B), *Pax6* (C, D), *LH2A/B* (E, F), *Lim1/2* (H, I), *Islet1* (K, L), and *Lbx1* (N, O) in neural tubes of chick embryos electroporated with *Lmx1b*-EGFP. All pictures are taken at stage 24, 48 h.a.e. Arrowheads point to domains of non-affected neuronal populations. Arrows point to neuronal populations affected by non-cell autonomous *Lmx1b* ectopic expression (as evident from F, I, L, O). G, J, M, P, Quantitative analysis of dI1–dI6 interneurons in embryos electroporated with *Lmx1b* and EGFP alone. Q, A diagram showing location of dorsal interneurons in non-electroporated and *Lmx1b*-electroporated chick spinal cord. RP, Roof plate; EP, electroporation.

slight increase in dI1 interneuron numbers within their endogenous domain was still observed (Fig. 5C, E). These data indicate that *Bmps* are required to allow *Lmx1a/b*-induced roof plate to repattern adjacent neural tube tissue in our overexpression assays. These data also suggest that there are other *Lmx1*-dependent factors that specifically positively regulate dI1 interneuron development, even when *Bmp* signaling is downregulated.

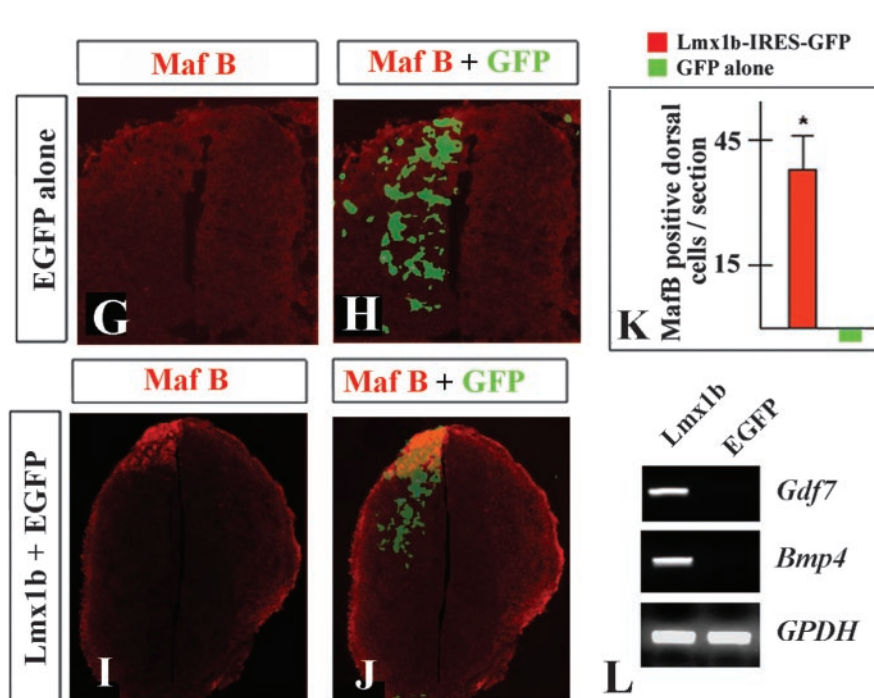
In contrast to *Bmp* inhibition, most *Lmx1a/b*-overexpression phenotypes were unaffected when either gene was coelectroporated with the *Wnt* inhibitor *dnWnt1*. The ventral extension of *Pax7* and *Msx1/2* expression domain and strong ventral shift of *Pax6* expression were still observed (Fig. 5G, H and data not shown). Additionally, no dI4–dI6 interneurons were detected on the electroporated sides of the neural tubes (Fig. 5J, L and data not shown). Downregulation of *Wnt* signaling when *Lmx1a/b* was overexpressed, however, had a very specific effect on the development of dI1 interneurons. Although dI1 interneurons



chick, 24 h.a.e.



dreher mouse, 24 h.a.e.



**Figure 4.** Lmx1b acts upstream of Lmx1a and can partially rescue roof plate phenotype in *dreher* mouse embryos. *A–C*, Expression of Lmx1a (*A, B*) and quantitative analysis of numbers of Lmx1a-positive cells (*C*) in chick neural tubes electroporated with Lmx1b 24 h.a.e. *D–F*, Expression of Lmx1b (*D, E*) and quantitative analysis of numbers of Lmx1b-positive cells (*F*) in chick neural tubes electroporated with Lmx1a 24 h.a.e. *G–K*, Expression of MafB (*G–J*) and quantitative analysis of number of MafB-positive cells (*K*) in developing spinal cords of *dreher* mouse embryos electroporated with Lmx1b (*I, J*) or EGFP alone (*G, H*) 24 h.a.e. *L*, RT-PCR analysis of expression of *Gdf7* and *Bmp4* in *dreher* developing spinal cords electroporated with Lmx1b and EGFP.

were significantly expanded ventrally in neural tubes coelectroporated with *Lmx1b* and *dnWnt1*, their increase in number was only moderate (25% increase) (Fig. 5*I, K*). This increase was much lower than that in neural tubes expressing exogenous *Lmx1b* alone (fourfold increase), indicating that downregulation of Wnt signaling partially suppressed the activity of *Lmx1a/b* to induce dI1 interneurons. Because Wnts have been reported previously to be mitogenic signals for dorsal neural tube cells (Megasson and McMahon, 2002), it was possible that the negative effect of *dnWnt1* on *Lmx1a/b*-dependent dI1 interneuron induction is attributable to an inhibition of cellular proliferation. Alternatively, Wnt signaling may regulate *Lmx1*-dependent dI1 interneuron specification more directly. To distinguish these possibilities, we evaluated the effects of *dnWnt1* on the proliferation of dorsal neural tube cells. When the *Lmx1b* and *dnWnt1* expression vectors (1  $\mu\text{g}/\mu\text{l}$  each) were coelectroporated, we usually observed a 70% decrease of BrdU incorporation in dorsal neural tubes compared with overexpression of *Lmx1b* alone. We duplicated this 70% reduction of BrdU incorporation by applying the cell cycle inhibitors olomoucine (200  $\mu\text{M}$ ) or aphidicolin (10  $\mu\text{M}$ ) on the top of *Lmx1b* (1  $\mu\text{g}/\mu\text{l}$ )-electroporated chick embryos (data not shown). Despite the presence of these inhibitors, *Lmx1b* overexpression still caused a twofold increase in the number of dI1 interneurons (Fig. 5*K*). Thus, a decrease of cell proliferation cannot fully account for the entire decrease in the number of Lmx1b-induced dI1 interneurons when Wnt signaling is downregulated. Instead, we conclude that Wnt1 has a more specific role in *Lmx1a/b*-dependent dI1 interneuron specification. To determine whether other signaling pathways have discernable roles in roof plate function, we coelectroporated *Lmx1b* or *Lmx1a* together with *noggin*, *folliculin*, and *dnWnt1*. No dI1 interneurons were detected in the majority of sections of these embryos. We therefore conclude that, in our overexpression assays, Bmps and Wnts act as the major components of *Lmx1*-dependent roof plate signaling. Bmp signaling influences the specification of numerous dorsal cellular populations, whereas Wnt signaling specifically regulates dI1 specification.

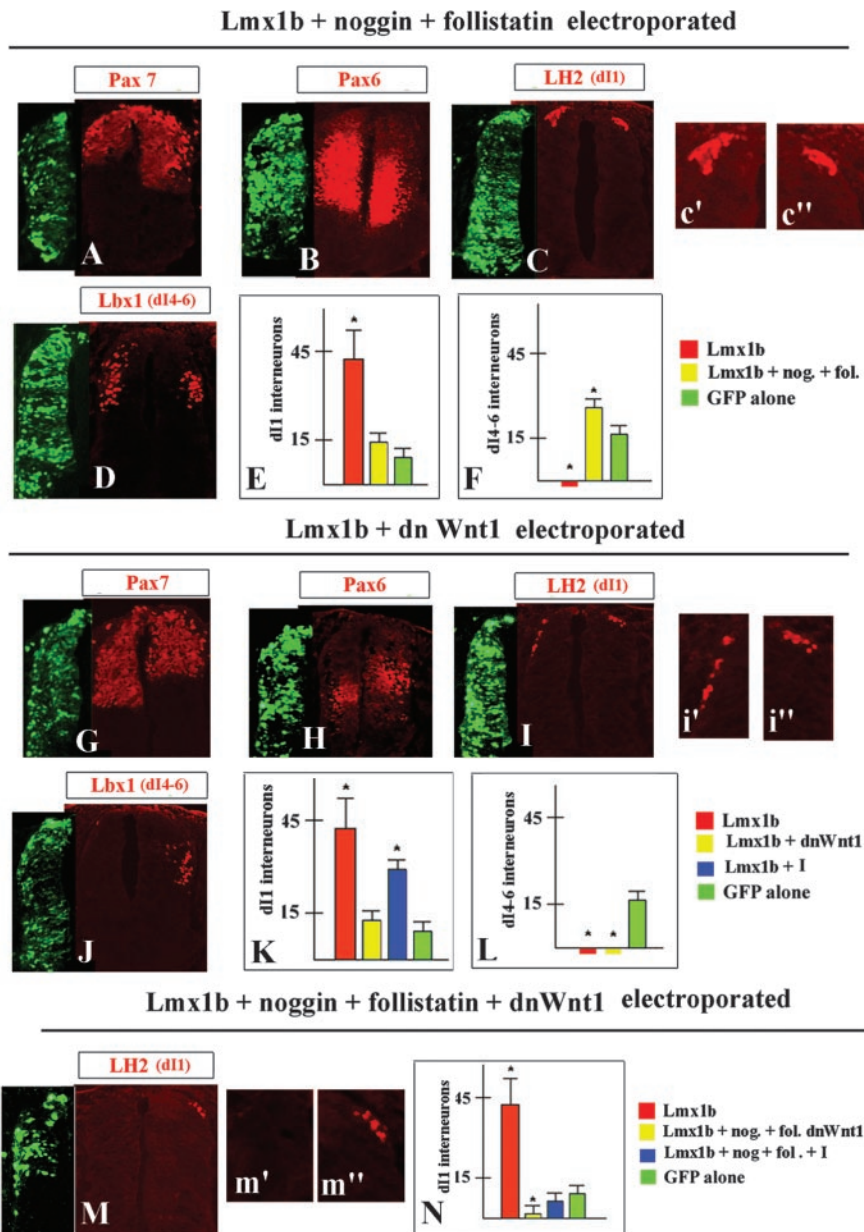
**Lmx1b is not involved in dI5 and dILB interneuron specification**

*Lmx1b* is expressed not only in roof plate but also in other cellular populations of the spinal cord, including dI5 and dILB

interneurons in both chick and mouse (Fig. 1E–G). To test whether *Lmx1b* is involved in specification and early differentiation of these neurons, we electroporated *Lmx1b* into stage 15 chick neural tubes, at the time of initiation of normal exogenous *Lmx1b* dI5 expression. To avoid the complications of any non-autonomous effects of ectopic *Lmx1b*-induced roof plate on dorsal interneuron cell fate, we limited our analysis to sections in which the expression of exogenous *Lmx1b* was restricted to intermediate regions of the neural tube (supplemental Fig. 3B,D, available at [www.neurosci.org](http://www.neurosci.org), and data not shown). Using LH2, Isl1, Lim1/2, Brn3a, and Lbx1 immunostaining, we found that *Lmx1b* overexpression neither induced nor repressed expression of any of these markers (supplemental Fig. 3A–D, available at [www.jneurosci.org](http://www.jneurosci.org), and data not shown). dI5 and dILB cell fates were also assessed in *Lmx1b* targeted null mutant mice (Chen et al., 1998). Using an *Lmx1b* *in situ* probe that detected both wild-type and mutant transcripts, we found no difference between wild-type and *Lmx1b*<sup>-/-</sup> embryos in either the number or distribution of dorsal *Lmx1b*-positive cells at E11.5 or E12.5 (data not shown). Immunohistochemical analysis revealed that dI5 and dILB neurons (and flanking dI1–dI4 and dI6 interneurons) were normally specified in *Lmx1b*<sup>-/-</sup> embryos (supplemental Fig. 3E–H, available at [www.jneurosci.org](http://www.jneurosci.org), and data not shown). Furthermore, there was no difference in the numbers of proliferating and apoptotic cells in dorsal and intermediate developing spinal cord of wild-type and *Lmx1b*<sup>-/-</sup> embryos at E10.5–E12.5. (data not shown). We conclude that *Lmx1b* has no role in the specification or early differentiation of dI5 and dILB interneurons.

### Mash1, normally expressed in intermediate neural tube progenitors, can suppress roof plate-inducing activity of *Lmx1b*

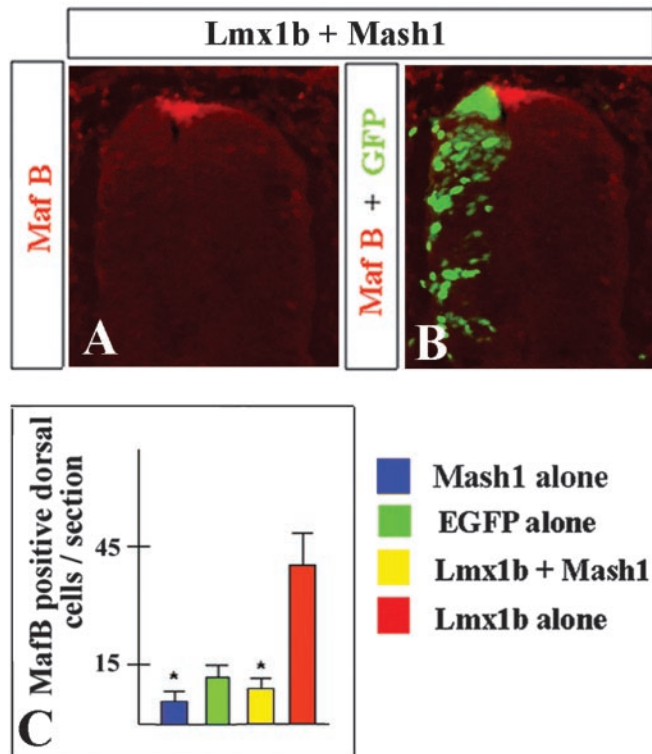
Our data indicate that, because overexpression of *Lmx1b* in stage 10 naive intermediate neural plate explants induces roof plate and prevents expression of dI5 interneuron markers, roof plate induction is the primary role of *Lmx1b* in the early developing chick spinal cord. At later stages, however, dI5 and dILB interneurons express endogenous *Lmx1b* in both chick and mouse without acquiring roof plate properties. This suggests that, during normal development, the roof plate-inducing activity of *Lmx1b* must become suppressed in intermediate cells and limited to the dorsal midline. Evidence of repression is also provided by our electroporation of *Lmx1b* into chick stage 10 neural plates. In these experiments, ectopic *Lmx1b*-induced roof plate was always limited to the most dorsal region of the neural tube and was never observed in intermediate spinal cord (Fig. 2A,B and data not shown). One



**Figure 5.** Blocking of *Lmx1b*-dependent roof plate signaling by Bmps and Wnts inhibitors. Effect of overexpression of *Lmx1b* together with *noggin* (*nog.*) and *follistatin* (*fol.*) (A–F), *Lmx1b* together with *dnWnt1* (G–L), and *Lmx1b* together with *noggin* and *follistatin*–*dnWnt1* (M, N) on expression of Pax7 (A, G), Pax6 (B, H), and dI1 (C, I) and dI4–dI6 (D, J) interneuron development. Insets show higher magnifications of selected neuronal populations. Quantitative analysis of numbers of selected classes of interneurons in neural tubes electroporated with *Lmx1b* together with *noggin* and *follistatin* (E, F), *Lmx1b* together with *dnWnt1* (K, L), and *Lmx1b* and together with *noggin* and *follistatin*–*dnWnt1* (N). The label I indicates cell cycle inhibitors amphidicolin and olomoucine.

factor that may suppress the roof plate-inducing activity of *Lmx1b* is Mash1. Mash1 (and its chick homolog Cash1) initiates its expression in the intermediate neural tube shortly after neural tube closure (Gowan et al., 2001) (data not shown). It has been implicated in the development of several neuronal lineages and is expressed in both dI5 and dILB progenitors before initiation of *Lmx1b* expression in these differentiating neurons (Gross et al., 2002; Muller et al., 2002; Qian et al., 2002). To test whether Mash1 could suppress the roof plate-inducing activity of *Lmx1b*, we coelectroporated *Mash1* and *Lmx1b* in caudal developing chick spinal cord just before neural tube closure at stage 10. In





**Figure 6.** Mash1 suppresses roof plate-inducing activity of Lmx1b. Expression of MafB (red) (*A*) alone and together with GFP (green) (*B*) in chick developing spinal cord coelectroporated with *Lmx1b* and *Mash1*, 24 h.a.e. Quantitative analysis of roof plate cells in embryos electroporated with *Mash1* alone versus *EGFP* alone and *Lmx1b* together with *Mash1* versus *Lmx1b* alone.

contrast to electroporation of *Lmx1b* alone, coelectroporation of *Lmx1b* with *Mash1* failed to induce ectopic roof plate, as detected by MafB, *Bmp4*, or *Gdf7* staining (Fig. 6 and data not shown). In addition, we observed an inhibition of endogenous roof plate formation when *Mash1* alone was electroporated into chick stage 10 neural plates (Fig. 6C and data not shown). Together, these data indicate that Mash1 can suppress roof plate-inducing activity of Lmx1b when overexpressed in chick developing spinal cord.

## Discussion

The roof plate is an important signaling center that regulates dorsal CNS patterning during vertebrate development, yet very little is understood about the molecular pathways that drive its induction and function. In this study, we show that *Lmx1b* can drive ectopic roof plate formation in the developing chick spinal cord and that Bmp and Wnt signaling are major components of Lmx1-dependent roof plate signaling. This function is not conserved across vertebrate evolution because *Lmx1b* is not expressed in the roof plate of mouse spinal cord. In mouse, caudal CNS roof plate formation relies entirely on *Lmx1a*. Lmx1b acts upstream of Lmx1a in the chick roof plate differentiation program and can partially rescue roof plate development in the *dreher* (*Lmx1a*<sup>-/-</sup>) mouse. Although *Lmx1b* is also normally expressed in dI5 and dILB interneurons in intermediate regions of the neural tube, loss-of-function or ectopic expression of *Lmx1b* does not alter the specification of these neurons. We therefore conclude that Lmx1b is not critically involved in dI5 and dILB interneuron specification and that roof plate induction is the primary activity of Lmx1b in early dorsal spinal cord development in chick. This activity may be suppressed by Mash1

(Cash1), normally expressed in intermediate regions of the mouse and chick neural tube.

## Roof plate induction mechanisms are different in chick and mouse caudal CNS

Roof plate is an embryonic signaling center that consists of non-dividing differentiated cells. In the developing vertebrate spinal cord, the roof plate forms from mitotically active progenitors at the lateral edges of the neural plate in response to Bmp signaling from adjacent ectodermal ectoderm (Lee and Jessell, 1999). Here we provide evidence that *Lmx1b* can induce ectopic expression of numerous roof plate markers, including Lmx1a, MafB, *Bmp4*, *Gdf7*, and *Wnt1*, when overexpressed in stage 10 chick neural plate. Surprisingly, Lmx1b was not sufficient to withdraw progenitor cells from the cell cycle, showing that it does not control this aspect of roof plate differentiation program. Nevertheless, we consider that Lmx1b can induce “roof plate,” because the induced structure non-autonomously affects the specification of adjacent neurons in the dorsal spinal cord. The results of our electroporation studies do not allow us to directly conclude that roof plate induction is the normal function of *Lmx1b* in chick developing spinal cord. However, together with the fact that Lmx1b is expressed in the chick roof plate progenitors and differentiated roof plate cells and that, similar to other roof plate markers, Lmx1b expression is responsive to Bmp signaling, our data strongly suggest that *Lmx1b* contributes to roof plate formation during normal chick spinal cord development.

We showed recently that *Lmx1a* also has roof plate-inducing activities in the chick neural plate and is regulated by Bmp signaling (Chizhikov and Millen, 2004). We conclude that, in the chick, Lmx1b and Lmx1a have at least partially redundant roof plate-inducing properties. However, because Lmx1b acts upstream of Lmx1a, at least some of the roof plate-inducing activity of Lmx1b may be mediated by Lmx1a.

In mouse caudal CNS, roof plate induction relies entirely on the action of Lmx1a, because Lmx1b is never expressed in roof plate progenitors or differentiated roof plate in the mouse spinal cord. Loss of *Lmx1a* function in the *dreher* (*Lmx1a*<sup>-/-</sup>) mutant mouse results in failure of roof plate development, which in turn, causes subsequent disruptions in the specification, patterning, and differentiation of adjacent dorsal sensory interneurons in the *dreher* spinal cord (Millonig et al., 2000). Interestingly, both chick and mouse *Lmx1b* can induce roof plate gene expression in the *dreher* spinal cord, partially rescuing the *Lmx1a* mutant phenotype, indicating that mouse Lmx1b still has roof plate-inducing properties. This finding is also supported by the observation that chick and mouse *Lmx1b* have equivalent properties in chick electroporation experiments. Importantly, however, mouse *Lmx1b* is not expressed in roof plate progenitors or developing roof plate, despite high levels of endogenous Bmp signals from the adjacent epidermal ectoderm, levels that are sufficient to induce *Lmx1a*. This indicates that the primary difference between chick and mouse *Lmx1b* is not its functional properties but rather the regulation of its expression. Cross-species and inter-loci comparative sequence analyses are likely to reveal regulatory differences contributing to this fundamental difference between mouse and chick roof plate-inductive mechanisms.

Although the roof plate-inducing activities of *Lmx1a* and *Lmx1b* are similar, they are not entirely overlapping. In contrast to *Lmx1a* (Chizhikov and Millen, 2004), *Lmx1b* overexpression cannot cause progenitor cell cycle withdrawal in either mouse explants or chick spinal cord. Thus, the cell cycle withdrawal activity is unique to Lmx1a. Whether these differences are attrib-



utable to actual sequence differences between Lmx1a and Lmx1b proteins or whether it is attributable to the requirement for different cofactors remains to be investigated.

### The molecular nature of Lmx1-dependent roof plate signaling

The ability of *Lmx1b* and *Lmx1a* to induce functional roof plate provided an opportunity to assess the molecular nature of Lmx1-dependent roof plate signaling. By coexpression of *Lmx1b* or *Lmx1a* together with inhibitors of Bmp and Wnt signaling in chick developing spinal cord, we showed that Bmps are major components of Lmx1a/b-dependent roof plate signaling in these experimental conditions, regulating the development of many dorsal interneurons. Interestingly, loss of roof plate Bmp signaling (but not Wnt signaling) observed in the *dreher* mouse spinal cord is also associated with decrease of dI1 interneuron numbers (Millen et al., 2004), suggesting that Bmps may be important mediators of Lmx1 signaling not only in the chick but also in the mouse. *Lmx1a/b* expression also activates Wnt signaling. In contrast to widespread actions of Bmps, the action of Lmx1-dependent Wnt signaling is limited to the positive regulation of dI1 interneuron development. Previous studies have identified Wnt1 as a mitogen for the neural cells (Megason and McMahon, 2002). We showed that the effects of cell cycle inhibitors on dI1 development in the presence of ectopic *Lmx1b* are not equivalent to coexpression of *Lmx1b* and Wnt1 inhibitors. These results indicate that Wnt1 plays a more direct role in patterning dI1 specification. Interestingly, overexpression of *Wnt1* in chick had no significant effect on the specification of any group of dorsal or intermediate cells except dI1 interneurons, suggesting that Wnt1 is unlikely a major regulator of dorsal cell identity in chick caudal CNS. This is in contrast to mouse, in which knock-out studies identified Wnt1 and Wnt3a as major regulators of dorsal cell identity in developing neural tube (Muroyama et al., 2002). Together, this suggests that there may be differences in the role of Wnt signaling in dorsal neuronal specification between chick and mouse.

### Lmx1b and dI5/dILB dorsal interneuron development

Our stage 10 neural plate electroporation data indicate that the primary function of Lmx1b in the early developing spinal cord is roof plate induction. At later stages, however, differentiating dI5 and dILB interneurons express endogenous Lmx1b in both chick and mouse without acquiring roof plate properties. By coelectroporating chick stage 15 neural tubes with *Lmx1b* and *Mash1*, which are normally expressed in intermediate neural tube including dI5 and dILB progenitors, we showed that *Mash1* can inhibit the roof plate-inducing activity of Lmx1b. Thus, *Mash1* (Cash1) may be one factor that prevents dI5 and dILB progenitors from adopting roof plate properties. Analysis of *Mash1*<sup>-/-</sup> mice is required to further support this hypothesis.

Interestingly, our loss-of-function studies in mouse revealed no role for Lmx1b in dI5 and dILB interneuron specification. This conclusion was further supported by gain-of-function experiments, performed on stage 15 chick developing spinal cord, at the time of initiation of expression of endogenous *Lmx1b* in dI5 interneurons. These data show that, at this stage, exogenous Lmx1b can neither induce additional dI5 interneurons nor block normal specification of other dorsal interneurons. This is in contrast to other *Lmx1b*-expressing neuronal populations in the CNS in which *Lmx1b* regulates cell fate [e.g., dopaminergic and serotonergic neurons (Smidt et al., 2000; Ding et al., 2003)]. Additional analysis of dI5/dILB neuronal migration and their axon

development is required to determine whether *Lmx1b* is involved in later steps of development of dI5 and dILB neurons.

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