



Published in final edited form as:

*J Neurosci.* 2009 September 9; 29(36): 11377–11384. doi:10.1523/JNEUROSCI.0969-09.2009.

## Overlapping function of *Lmx1a* and *Lmx1b* in anterior hindbrain roof plate formation and cerebellar growth

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### Abstract

The roof plate is an organizing center in the dorsal central nervous system that controls specification and differentiation of adjacent neurons through secretion of the BMP and WNT signaling molecules. *Lmx1a* is expressed in the roof plate and its progenitors at all axial levels of the CNS and is necessary and sufficient for roof plate formation in the spinal cord. In the anterior CNS, however, a residual roof plate develops in the absence of *Lmx1a*. *Lmx1b*, a highly related gene required for the development of the isthmic organizer, is co-expressed with *Lmx1a* in dorsal rhombomere 1 roof plate. Although *Lmx1b* null mice do not show a substantial deficiency in hindbrain roof plate formation, *Lmx1a/Lmx1b* compound null mutants fail to generate hindbrain roof plate. This observation indicates that both genes act in concert to direct normal hindbrain roof plate formation. Since the requirement of *Lmx1b* function for normal isthmic organizer at the mid-hindbrain boundary complicates analysis of a distinct dorsal patterning role of this gene, we also used a conditional knockout strategy to specifically delete dorsal midline *Lmx1b* expression. Phenotypic analysis of single and compound conditional mutants confirmed overlapping roles for *Lmx1* genes in regulating hindbrain roof plate formation and growth and also revealed roles in regulating adjacent cerebellar morphogenesis. Our data provides the first evidence of overlapping function of the *Lmx1* genes during embryonic CNS development.

### Keywords

dorsal; patterning; cerebellum; hindbrain; genetics; development

### INTRODUCTION

During hindbrain development, the isthmic organizer (IsO) at the mid/hindbrain junction determines anterior/posterior patterning signals (Sillitoe and Joyner, 2007) while the 4<sup>th</sup> ventricle roof plate provides dorsal positional information (Chizhikov et al., 2006). The hindbrain roof plate differentiates into the epithelium of the choroid plexus, a structure with

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multiple physiological functions including the secretion of cerebrospinal fluid (Curre et al., 2005). The genetic cascades driving IsO formation and function have been extensively studied (Liu and Joyner, 2001; Wang and Zoghbi, 2001; Wurst and Bally-Cuif, 2001; Nakamura et al., 2005). Far less is understood regarding hindbrain roof plate and choroid plexus formation.

Previously, roof plate studies have primarily concentrated on the spinal cord, where roof plate progenitors are induced at the lateral edges of the neural plate through a Bmp-Lmx1a signaling pathway (Liem et al., 1997; Lee et al., 1998; Lee and Jessell, 1999; Lee et al., 2000; Chizhikov and Millen, 2004a, 2005). Although loss of *Lmx1a* completely abolishes roof plate induction in the spinal cord, a residual roof plate still forms in rhombomere 1 (rh1), the most anterior segment of the hindbrain in *Lmx1a*<sup>-/-</sup> (*dreher*) mice. We hypothesized that *Lmx1b*, a protein with 64% amino acid identity to mouse *Lmx1a* (100% identity in the homeodomain and 67% and 83% in each LIM domain), functions redundantly with *Lmx1a*. Although *Lmx1b* is expressed in the IsO, it is also expressed in the anterior hindbrain roof plate, in a domain which closely correlates with the residual roof plate in *Lmx1a*<sup>-/-</sup> embryos suggesting redundant function to *Lmx1a*. Further, *Lmx1b* can induce roof plate when overexpressed in chick spinal cord (Chizhikov and Millen, 2004b). The function of *Lmx1b* in the developing IsO is well characterized (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). The specific role of *Lmx1b* in hindbrain roof plate development, however, has never been assessed.

Here we show that *Lmx1a* and *Lmx1b* are individually dispensable for hindbrain roof plate induction. Complete loss of both genes, however, abolishes hindbrain roof plate demonstrating that both genes have overlapping roles in inducing this critical embryonic signaling center. Since *Lmx1b* is required for IsO maintenance (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007), the loss of IsO in the *Lmx1b* null background complicated our analysis. To isolate the anterior/posterior patterning functions from the dorsal/ventral patterning role of *Lmx1b*, we used a conditional knockout strategy to specifically delete *Lmx1b* in the dorsal midline without disturbing its isthmic expression. These *Lmx1b* conditional mutant mice were also bred with *Lmx1a* mutant mice to further reduce *Lmx1* gene dosage. *Lmx1a*; *Lmx1b* conditional compound mutant mice showed severe 4<sup>th</sup> ventricle roof plate size reduction, confirming overlapping roles for the dorsally expressed *Lmx1* genes in roof plate formation. The viability of these compound conditional mutant mice with small roof plate further allowed us to demonstrate that rh1 roof plate directs multiple aspects of cerebellar morphogenesis, including cerebellar anlage proliferation. Together, these experiments provide the first evidence for overlapping function of *Lmx1a* and *Lmx1b* in the central nervous system.

## MATERIALS AND METHODS

### Mice

*Lmx1a* null (*Lmx1a*<sup>-/-</sup>) (*Lmx1a*<sup>dr-J</sup> Jackson Laboratories strain #000636), *Lmx1b* null (*Lmx1b*<sup>-/-</sup>) (Chen et al., 1998), *Lmx1b* floxed (*Lmx1b*<sup>flf</sup>) (Zhao et al., 2006), and *Lmx1a*-cre transgenic mice (Chizhikov et al., 2006) were genotyped as previously described. Cre activity was discerned by crossing *Lmx1a*-cre transgenic mice to ROSA26 floxed LacZ reporter mice (129S-Gt(ROSA)26Sortm1Sor/J; Jackson Laboratories; strain #003310) (Soriano, 1999). Compound conditional mutant mice (*Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mice) were generated by intercrossing *Lmx1b*<sup>flf</sup>; *Lmx1a*<sup>+/-</sup> mice and *Lmx1a*-cre; *Lmx1a*<sup>+/-</sup>; *Lmx1b*<sup>+/-</sup> mice. Noon of the observation of the vaginal plug was considered as embryonic day (E) 0.5.

The *Lmx1a*-cre BAC transgene includes wild-type exon 2, necessitating adjustment of the standard exon 2 *Lmx1a* genotyping protocol (Chizhikov et al. 2006) in mice carrying the transgene. Restriction analysis of the wild-type exon 2 amplified allele using HpyCh4V yielded 127bp, 14bp, 51bp, 33bp in 5' to 3' order. The *dr-J* point mutation abolishes the HpyCh4V site between the 51bp and 33bp fragments resulting in a 84 bp fragment. Restriction digests of the

exon 2 amplicon from *Lmx1a*<sup>-/-</sup> mice carrying the *Lmx1a*-cre transgene produced the 84bp *dr-J* band and the wild-type 51bp band. To distinguish these mice from heterozygous *Lmx1a*<sup>+/-</sup> mice carrying the *Lmx1a*-cre transgene, we compared the band intensities of the 51bp wild-type vs. 84bp mutant band. Genotyping conditions and primers are available upon request. All mice were maintained on mixed genetic backgrounds. All mouse procedures followed the policies of the University of Chicago and NIH Guidelines on Care and Use of Laboratory Animals.

### Tissue analysis

*In situ* hybridization was performed as previously described (Chizhikov et al., 2006). Probes used were mouse *Lmx1a*, *Lmx1b*, *Gdf7* (T. Jessell), *Wnt1* (A. McMahon), *Math1* (J. Johnson), *Ptf1a* (C. Wright), and *Otp* (A. Simeone). For the *Lmx1b* homeodomain-specific probe, exons 4, 5, and 6 were PCR amplified and cloned into PCRII-TOPO (Invitrogen), linearized with EcoRV, and antisense transcribed with SP6. Immunohistochemistry and BrdU analysis was performed as previously described (Chizhikov et al., 2006). Primary antibodies used were: *Lmx1a* (M. German), *Lmx1b* (T. Perlmann), BrdU (DSHB and Abcam), and Calbindin (Swant). Appropriate species-specific secondary antibodies were used (Jackson Immunological). X-gal staining and histology were performed as previously described (Chizhikov et al., 2006).

### Measurements and statistical analysis

All embryos were photographed on a LEICA MZFLIII microscope using a Spot II camera. The areas of 4<sup>th</sup> ventricle roof plate were measured using the Spot version 4.6 software in whole mount embryos. Three individual embryos of each genotype were measured three times (unit  $\mu\text{m}^2$ ) to obtain an average. Areas of sagittal sections of the cerebellar vermis were measured using four individual mice of each genotype. For quantitative analysis of the BrdU positive cells, three mice of each genotype were examined. The mitotic index of the cerebellar ventricular zone was calculated as the number of BrdU positive cells divided by the total number of cells (DAPI) in a set area (a total of 500 DAPI cells along the ventricular zone,  $100\mu\text{m} \times 10\mu\text{m}$  in area, were counted). Two tailed t-tests were used to determine statistical significance. \* indicates  $p < 0.01$ , \*\* indicates  $p < 0.001$ .

## RESULTS

### *Lmx1a* and *Lmx1b* are expressed in the rh1 roof plate and its derivative, the choroid plexus

Extending previous studies from chick (Adams et al., 2000) and zebrafish (O'Hara et al., 2005; Elsen et al., 2008), we carefully examined the developmental expression profile of *Lmx1b* in dorsal rh1 of the developing mouse (Fig. 1). We first observed *Lmx1b* expression at E8.0, along the lateral edges of the closing neural plate (Fig. 1B), the region that gives rise to rh1 roof plate (Awatramani et al., 2003; Chizhikov et al., 2006; Hunter and Dymecki, 2007). After neural tube closure, *Lmx1b* was highly expressed in rh1 roof plate and its later derivative, the choroid plexus epithelium, at all stages examined (Fig. 1D,G,J and data not shown). In the mid/hindbrain region, we also observed *Lmx1b* expression in the IsO (Fig. 1D arrow), where *Lmx1b* has a critical function in IsO maintenance (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). Similar to *Lmx1b*, *Lmx1a* was expressed at the lateral edges of the mid/hindbrain neural plate but its expression was initiated slightly later than *Lmx1b*, at E 8.5 (Failli et al., 2002; Millen et al., 2004) (Fig. 1A). *Lmx1a* was also expressed in rh1 roof plate and choroid plexus epithelium during embryonic and postnatal development (Fig. 1C,F and (Chizhikov et al., 2006)). However, beginning from E12.5, *Lmx1a* expression was further initiated in the adjacent rhombic lip (Fig. 1F arrow). In contrast, *Lmx1b* expression remained restricted to the choroid plexus at both embryonic and postnatal stages (Fig. 1G,H,J). Given

that both genes have overlapping expression domains, we hypothesized that both are involved in rh1 roof plate development and may function together.

### ***Lmx1a* and *Lmx1b* have overlapping roles in hindbrain roof plate development**

Although the role of *Lmx1b* in IsO is well documented (Adams et al., 2000; Matsunaga et al., 2002; O'Hara et al., 2005; Guo et al., 2007), its specific role in rh1 roof plate development has never been assessed. We therefore examined rh1 roof plate in *Lmx1b* null (*Lmx1b*<sup>-/-</sup>) mice. Despite considerable disruption of the mid/hindbrain junction caused by loss of *Lmx1b* function in the IsO (Adams et al., 2000; Matsunaga et al., 2002; O'Hara et al., 2005; Guo et al., 2007), *Lmx1b*<sup>-/-</sup> embryos had a relatively normal rh1 roof plate based on its size, shape and normal expression of multiple roof plate markers, including *Gdf7*, *Wnt1*, and *Msx1* at E10.5 (n=3 for each marker) (Fig. 2C,D and data not shown). These data indicate that despite its significant role in IsO maintenance, *Lmx1b* is largely dispensable for rh1 roof plate development. This is in contrast to *Lmx1a* since its loss results in severe reduction of rh1 roof plate (Fig. 2E,F, (Millonig et al., 2000; Millen et al., 2004; Chizhikov et al., 2006)).

To test if *Lmx1b* cooperates with *Lmx1a* during roof plate development, we next examined the rh1 roof plate phenotype of double null (*Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup>) mutant embryos. Strikingly, at E10.5, expression of *Gdf7* was completely missing or extremely reduced in double null mutant embryos (Fig. 2G) (n=3). In addition, we detected abnormal expression of *Wnt1* in dorsal rh1 of E10.5 *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos (n=3). In wild-type embryos, *Wnt1* is expressed in the rhombic lip adjacent to hindbrain roof plate (Awatramani et al., 2003; Hunter and Dymecki, 2007). We confirmed this expression in rh1 of wild-type embryos (Fig. 2B,J arrowheads), but observed fused medial expression in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos at E10.5 (Fig. 2H,K arrow), further demonstrating the lack of rh1 roof plate.

We next tested whether dorsal midline cells in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos retained any properties of roof plate. We and others have shown that rh1 roof plate signaling is required to induce *Math1* in the adjacent cerebellar rhombic lip (Alder et al., 1999; Chizhikov et al., 2006). In *Lmx1b*<sup>-/-</sup> mice, despite IsO disruption, rh1 *Math1* expression was similar to wild-type embryos (Fig. 3B). In *Lmx1a*<sup>-/-</sup> mice, although the roof plate was small, *Math1* was still induced (Fig. 3C). However, no *Math1* expression was detected in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> mice (n=4) (Fig. 3D) indicating complete loss of roof plate signaling. Importantly, *Ptf1a*, a marker of cerebellar ventricular zone whose induction is not dependent on roof plate signaling (Chizhikov et al., 2006), was still present in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos (Fig. 3H). This indicates that the cerebellar ventricular zone which forms just ventral to the rhombic lip within dorsal rh1 was still present. *Ptf1a* expression was reduced however, since the roof plate is required to expand the ventricular zone progenitor pool that expresses this marker (Chizhikov et al., 2006). At an earlier stage (E10.5), *Otp*, a ventral marker, was expressed normally in all mutant embryos (Fig. 3I-L), arguing that there was no perturbation of the alar/basal plate boundary in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos and that dorsal/ventral patterning phenotype in rh1 phenotype restricted to very dorsal cell types. This confirms our previous finding that roof plate-dependent cell fate specification is limited to just the adjacent rhombic lip in rh1 (Chizhikov et al., 2006). Together, both our morphological and functional data indicate that *Lmx1b* and *Lmx1a* have overlapping roles in the development of rh1 roof plate.

### **Generation of mice with conditional loss of *Lmx1b* only in the roof plate where it is co-expressed with *Lmx1a***

Our analysis of *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos suggested involvement of *Lmx1b* in rh1 roof plate development. The mid/hindbrain region of *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> mice however, was severely mispatterned since *Lmx1b* expression at the mid/hindbrain is required to maintain the IsO (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). This anterior/posterior

mis patterning significantly complicated our assessment of the role of *Lmx1b* in rh1 roof plate formation. To more specifically examine the role of *Lmx1b* just in roof plate development, we designed a conditional knockout strategy to delete *Lmx1b* only in the dorsal midline, without disturbing its expression in the IsO.

The mice used to create the desired mutant embryos carried a floxed allele of *Lmx1b* (*Lmx1b<sup>f</sup>*) (Zhao et al., 2006) and a null allele of *Lmx1b* (*Lmx1b<sup>-</sup>*) (Chen et al., 1998). In the deleter mouse strain used, cre recombinase expression was controlled by *Lmx1a* regulatory elements (*Lmx1a-cre*) (Fig. 4A) (Chizhikov et al., 2006). A series of control experiments were initially conducted to determine the efficacy of the strategy. First, we verified that the *Lmx1b<sup>f</sup>* allele was not hypomorphic by assessing postnatal (P) 20 cerebellar morphology in *Lmx1b<sup>f/-</sup>* mice, since adult cerebellar morphology is sensitive to perturbations of IsO (Sato et al., 2004; Sillitoe and Joyner, 2007). As expected, we observed normal cerebellar morphology in *Lmx1b<sup>f/-</sup>* mutants, demonstrating that embryonic IsO was undisturbed (data not shown). We next verified cre activity in rh1 roof plate by assessing LacZ activity in embryos from transgenic *Lmx1a-cre* mice mated with ROSA26 floxed LacZ reporter mice (Soriano, 1999). Weak LacZ activity was initially detected at E9.0, 0.5 days later than endogenous *Lmx1a* expression and approximately 1 day later than endogenous *Lmx1b* expression (Fig. 4B). By E9.5, however, robust LacZ activity was readily detected in the majority of rh1 roof plate cells (Fig. 4C).

To specifically delete the dorsal expression domain of *Lmx1b*, we crossed *Lmx1b<sup>f/-</sup>* mice with *Lmx1b<sup>+/-</sup>* mice carrying the *Lmx1a-cre* transgene (the *Lmx1b<sup>f/-</sup>;Lmx1a-cre* genotype referred to as *Lmx1b<sup>cko/-</sup>*). To verify that *Lmx1b* expression was eliminated only in the roof plate in *Lmx1b<sup>cko/-</sup>* double mutant mice, we conducted *in situ* hybridization using a probe specific for the exons flanked by loxP sites. As expected, *Lmx1b* expression was detected in the IsO and rh1 roof plate of E10.5 wild-type embryos (Fig. 4D). In *Lmx1b<sup>cko/-</sup>* embryos, we detected normal expression of *Lmx1b* in the IsO but none in the rh1 roof plate (Fig. 4E). Transverse sections through the rh1 further verified that expression in 4<sup>th</sup> ventricle roof plate was specifically deleted in *Lmx1b<sup>cko/-</sup>* embryos (Fig. 4 F-H). Finally, we assessed the roof plate phenotype of *Lmx1b<sup>cko/-</sup>* mice. The normal rh1 roof plate in these embryos (data not shown) confirmed our earlier observation in *Lmx1b<sup>-/-</sup>* embryos that partial or complete loss of *Lmx1b* alone has minimal effect on rh1 roof plate development.

### Both *Lmx1* genes are required for normal rh1 roof plate growth

To further reduce hindbrain roof plate *Lmx1* gene dosage we crossed *Lmx1b<sup>cko/-</sup>* mice with *Lmx1a<sup>-/-</sup>* mice, to generate *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* double mutants. At E10.5, we observed dramatic abnormalities in 4<sup>th</sup> ventricle roof plate in *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* embryos (n=4). Patches of *Gdf7*-negative medial cells, not found in *Lmx1a<sup>-/-</sup>* or wild-type embryos were detected (Fig. 5C, arrowheads). Additionally, 4<sup>th</sup> ventricle roof plate size was significantly smaller in *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* mice than *Lmx1a<sup>-/-</sup>* and wild-type mice at E10.5 (Fig. 5D), although the size was equivalent at E9.5 in *Lmx1a<sup>-/-</sup>* and *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* mice (Fig. 5 B',C'). Notably, the E10.5 conditional double mutant phenotype was not as severe as that observed in *Lmx1a<sup>-/-</sup>;Lmx1b<sup>-/-</sup>* double null mutants (compare Fig. 2 G,H with 5C). We predicted that this was due to transient, residual expression of *Lmx1b* in these conditional mutants since LacZ expression in *Lmx1a-cre;ROSA* embryos, demonstrated that cre-activity in the roof plate was only robust from E9.5. Indeed, at E9.5, we observed residual *Lmx1b* expression in *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* double mutant mice (Fig. 4I-L). Thus, our analysis of *Lmx1a<sup>-/-</sup>;Lmx1b<sup>cko/-</sup>* embryos show that *Lmx1b* expression from E8.0 until at least E9.5, even in the absence of *Lmx1a*, is sufficient to normally induce rh1 roof plate. Our analysis of these double conditional mutants however also revealed partially overlapping roles for *Lmx1* genes in later regulation of hindbrain roof plate growth.

### ***Lmx1* dependent roof plate signaling is required for normal cerebellar growth and patterning**

We next examined cerebellar anlage patterning in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> embryos. Hybridization with a *Wnt1* *in situ* probe determined that the IsO was still intact (Fig. 5E). Additionally, *Math1* was still expressed in the developing cerebellar anlage of the *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> embryos (Fig. 5F), indicating that the severely reduced 4<sup>th</sup> ventricle roof plate was still able to initiate rhombic lip development. *Ptf1a*, expressed in the ventricular zone of the developing cerebellar anlage, was also still expressed relatively normally in the *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> embryos (Fig. 5G, arrowheads). Thus we conclude that despite dramatic roof plate defects, early embryonic cerebellar anlage patterning is not markedly affected in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> embryos.

To date, it has been difficult to comprehensively define the role of rh1 roof plate signaling on adjacent cerebellar morphogenesis. Previously we used a diphtheria toxin ablation strategy to demonstrate that rh1 roof plate signaling regulates adjacent cerebellar anlage proliferation, cell fate specification and patterning (Chizhikov et al., 2006). However *Gdf7-DTA* roof plate ablated mice do not survive past E12.5, long before the completion of the cerebellar morphogenesis. In addition, cerebellar anlage abnormalities in *Gdf7-DTA* mice were confounded by an open neural tube defect (Monuki et al., 2001; Chizhikov et al., 2006). Importantly, *Lmx1a*<sup>-/-</sup> and *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice survive past weaning making it possible to analyze the consequences of graded roof plate reduction on later stages of cerebellar morphogenesis.

Since our mutations exist on a mixed genetic background, we first developed a grading system to assess the variable adult *Lmx1a*<sup>-/-</sup> cerebellar phenotypes generated in this study (Suppl. Fig. 1). We assigned each individual *Lmx1a*<sup>-/-</sup> cerebellum to one of five phenotypic groups. Group 1 cerebella were nearly normal, while those in group 5 were poorly developed. This system allowed us to establish a quantitative means of assessing the phenotypic effects of loss of dorsal *Lmx1b* expression on the *Lmx1a* mutant background. While the cerebellar phenotypes of *Lmx1a*<sup>-/-</sup> mice were almost equally distributed between our 5 phenotypic groups, all *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice analyzed in this study (n=8) had very severe cerebellar defects and were assigned to groups 4 and 5, a significant skewing of severity (P<0.017) (Fig. 6C,F). The phenotypic severity of *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice was confirmed by reduced cerebellar vermis area based on mid-sagittal sections (p<0.001) (Fig. 6G). Despite the severe foliation patterning disruptions, Purkinje and granule cells were still present and a relatively normal cerebellar lamination pattern was evident in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice (Fig. 6F). However, in two of these mice, we detected ectopic Purkinje and granule cells anterior to the cerebellum within the inferior colliculus of the midbrain (Fig. 6H-K). Together, these data indicate that *Lmx1*-dependent rh1 roof plate signaling is critical for establishing the proper size and appropriate three-dimensional structure of the adult cerebellum.

### **Graded loss of roof plate signaling results in reduced embryonic cerebellar anlage proliferation**

Since we have previously demonstrated that roof plate signaling drives adjacent cerebellar anlage proliferation (Chizhikov et al., 2006), we predicted that the size reduction of adult cerebella in *Lmx1a*<sup>-/-</sup> and *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice would correlate with reduced cell division in the cerebellar anlage due to reduced roof plate size. BrdU labeling at E12.5 demonstrated graded reduction in proliferation in the ventricular zone of the mutants compared to wild-type (Fig. 7). Therefore, our data suggests that reduced proliferation in early cerebellar anlage mediated by reduced roof plate size significantly contributes to the reduced cerebellar size in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice.

## DISCUSSION

### *Lmx1b* have overlapping function to *Lmx1a* in rh1 roof plate formation

To test the hypothesis that *Lmx1* genes have overlapping functions in the development of the hindbrain roof plate, we compared the embryonic roof plate phenotypes of *Lmx1a*<sup>-/-</sup>, *Lmx1b*<sup>-/-</sup>, and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>-/-</sup> mice. We showed that roof plate development is largely normal in *Lmx1b*<sup>-/-</sup> mutants, indicating no unique role for *Lmx1b* in hindbrain roof plate development. However, we observed complete deletion of the hindbrain roof plate in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>-/-</sup> double null mutants. This conclusion is based not only on loss of all roof plate markers in double null embryos, but also our demonstration that there is complete loss of roof plate function in these embryos. No induction of *Math1*, a marker of rhombic lip, occurs. Our data therefore provides genetic proof that *Lmx1b* function is overlapping to *Lmx1a* in roof plate development.

*Lmx1b* is expressed in the IsO where it is required to maintain the IsO, an important anterior-posterior signaling center at the mid/hindbrain junction (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). Thus, *Lmx1b*<sup>-/-</sup> and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>-/-</sup> double null mutants had disrupted mid/hindbrain patterning along the anterior/posterior axis. Others have shown that levels of *Fgf* signaling from the IsO influence anterior rh1 roof plate (Alexandre et al., 2006; Basson et al., 2008). Anterior rh1 roof plate lies adjacent to the IsO and occupies a narrow medial domain while the posterior rh1 roof plate widens over the expanse of the fourth ventricle (Chizhikov et al., 2006). The only roof plate phenotype that we observed in *Lmx1b*<sup>-/-</sup> mice was loss of the anterior rh1 roof plate. Posterior rh1 roof plate was unaffected in these mice. We conclude that the anterior roof plate defect is a direct result of loss of *Lmx1b*-dependent *Fgf* 8 IsO expression and not *Lmx1b* dorsal roof plate expression, since *Lmx1b* is required to maintain *Wnt1* IsO expression, which in turn is required for IsO *Fgf8* maintenance (Adams et al., 2000; Matsunaga et al., 2002).

To dissect dorsal roof plate function of *Lmx1b* from IsO function of this gene, we used *Lmx1a-cre* mice (Chizhikov et al., 2006) to delete a floxed *Lmx1b* allele just in the roof plate. Notably, the roof plate phenotype of *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mice was less severe than the *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>-/-</sup> mice. Two possibilities can explain this observation. Either *Lmx1b* IsO has a role in roof plate formation or there is a delay in excision of *Lmx1b* in the roof plate in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mice. Since the roof plate is near normal in *Lmx1b* null mice, our data strongly argue that the IsO plays no role in development of most of the 4<sup>th</sup> ventricle roof plate. Rather our data suggest that a delay in excision of dorsal midline *Lmx1b* is a more probable explanation of the intermediate roof plate phenotype and that transient expression of *Lmx1b* in the double conditional mutant embryos is sufficient to induce roof plate even in the absence of *Lmx1a*. Notably, although the roof plate was present, *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mice had a smaller 4<sup>th</sup> ventricle roof plate compared to *Lmx1a*<sup>-/-</sup> mice at E10.5, revealing overlapping roles for these genes in roof plate growth and confirming that dorsal *Lmx1b* on the dorsal midline acts together with *Lmx1a* to direct normal roof plate formation.

Our mutant analyses demonstrate that *Lmx1b* function is completely redundant to *Lmx1a* in roof plate development. However, the reverse is not true. *Lmx1a* has both unique and overlapping function with *Lmx1b*. We speculate that structural differences in these two highly related proteins may lead to differences in DNA-binding or co-factor interactions which may drive these functional differences.

### Cerebellar morphogenesis is dependent on redundant, *Lmx1*-dependent roof plate signaling

Based on our analysis of *Lmx1a*<sup>-/-</sup> mice, we demonstrated that reduced signaling from the small roof plate contributes to the small and mispatterned cerebellar phenotype in adult mice

(Millonig et al., 2000). Here we have shown that the adult *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> cerebellum was even smaller than that of *Lmx1a*<sup>-/-</sup> mutants. We did not observe gross abnormalities of the E12.5 cerebellar anlage patterning in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos. However, we observed smaller 4<sup>th</sup> ventricle roof plate in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos. We have previously demonstrated that signaling from rh1 roof plate controls cerebellar anlage proliferation (Chizhikov et al., 2006). Indeed, BrdU analysis confirmed that the small rh1 roof plate in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos resulted in dramatically reduced proliferation within the cerebellar anlage at E12.5, where neither *Lmx1* gene is expressed. Thus, although the roof plate and choroid plexus may have additional signaling roles later during cerebellar development, we hypothesize that the small adult cerebellar size in the *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mutants is partially attributable to an early growth failure of the mutant cerebellar anlage.

Although *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> mice had a very small cerebellum, cerebellar lamination was essentially normal in these mutants, indicating that although reduced in number, most cerebellar cell types were still generated and migrated to appropriate final positions. We did, however, detect ectopic midbrain Purkinje cells in these mice which were never observed in *Lmx1a*<sup>-/-</sup> animals. Since *Lmx1b* expression was restricted to the rh1 roof plate and its derivative, the choroid plexus epithelium during embryogenesis and early post-natal development, our data indicate that roof plate signaling regulates positioning of the cerebellar cells.

### ***Lmx1* gene redundancy – more than just the roof plate?**

Our study provides the first detailed description of the unique and overlapping functions of *Lmx1a* and *Lmx1b* in the developing dorsal CNS where these genes are co-expressed. Notably, there are other regions of the CNS where both *Lmx1* genes likely interact. *Lmx1a* and *Lmx1b* are co-expressed in ventral midbrain dopaminergic (DA) neuronal progenitors. *Lmx1a*<sup>-/-</sup> mice have reduced numbers of midbrain DA neurons (Ono et al., 2007), and RNAi experiments in chick have demonstrated that *Lmx1a* is required by DA neuron progenitors (Andersson et al., 2006). *Lmx1b*<sup>-/-</sup> mice have DA neurons with different molecular expression, indicating that these cells are not properly differentiated (Smidt et al., 2000). Our demonstration of genetic interactions between *Lmx1a* and *Lmx1b* in the dorsal hindbrain suggests that detailed analysis of ventral midbrain development in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>-/-</sup> mice will reveal novel and severe DA abnormalities. There may also be *Lmx1* genetic redundancy in the developing inner ear where both genes are expressed and *Lmx1a* has been shown to be expressed and *Lmx1a* has a critical developmental role (Huang et al., 2008; Nichols et al., 2008).

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **ACKNOWLEDGEMENTS**

We would like to thank Michael German, Tom Jessell, Jane Johnson, Andrew McMahon Thomas Perlmann, Antonio Simeone, and Chris Wright for providing reagents. This work was supported by NIH grant RO1 NS044262 to KJM.

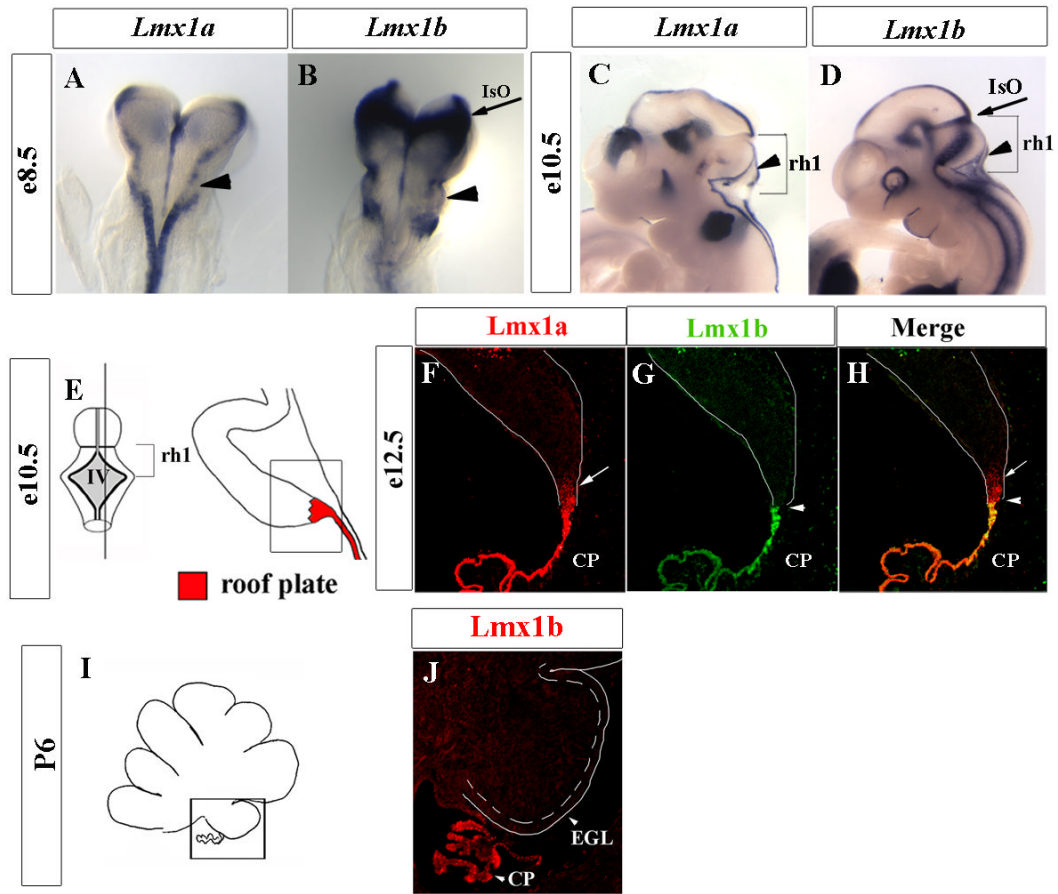
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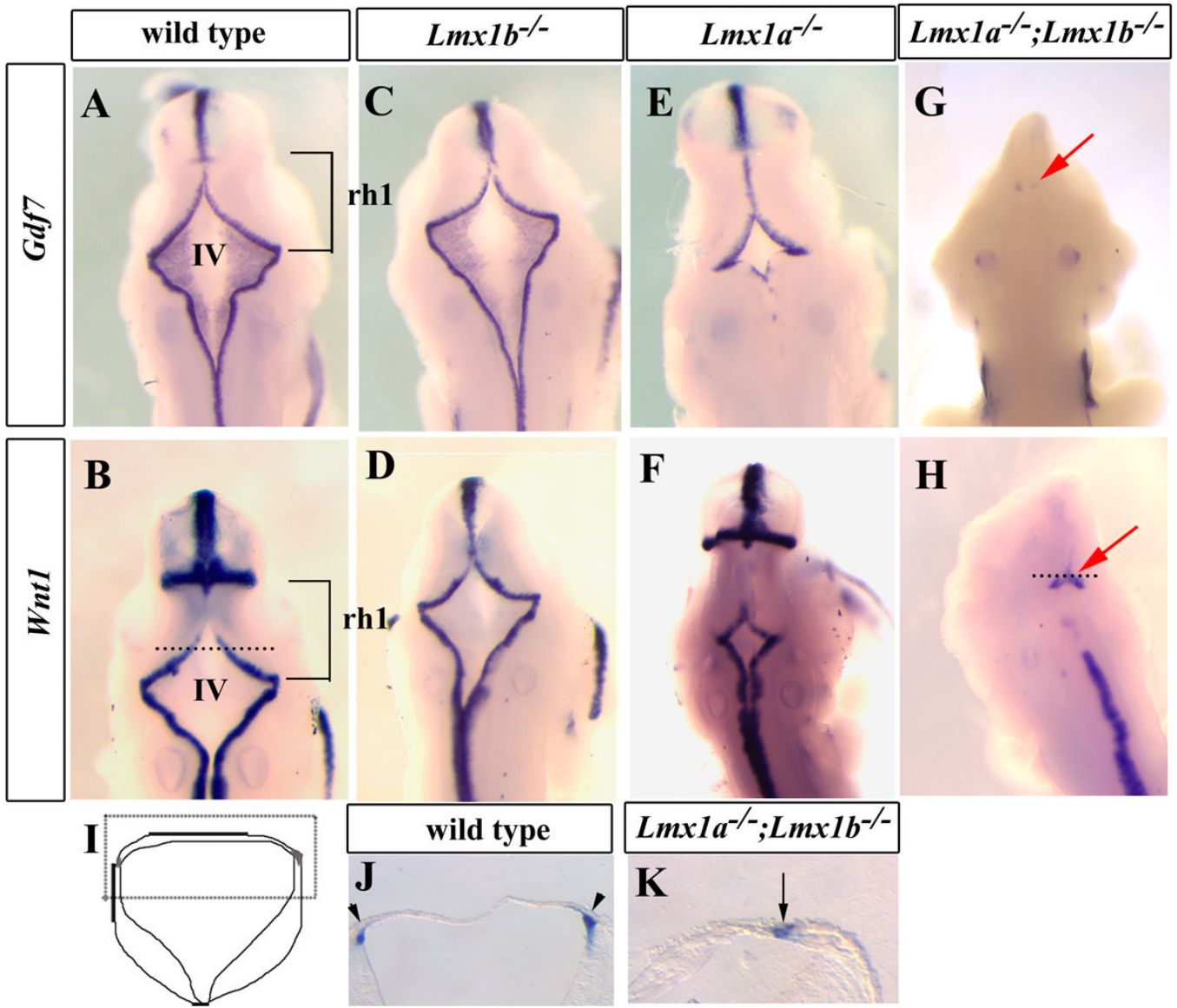
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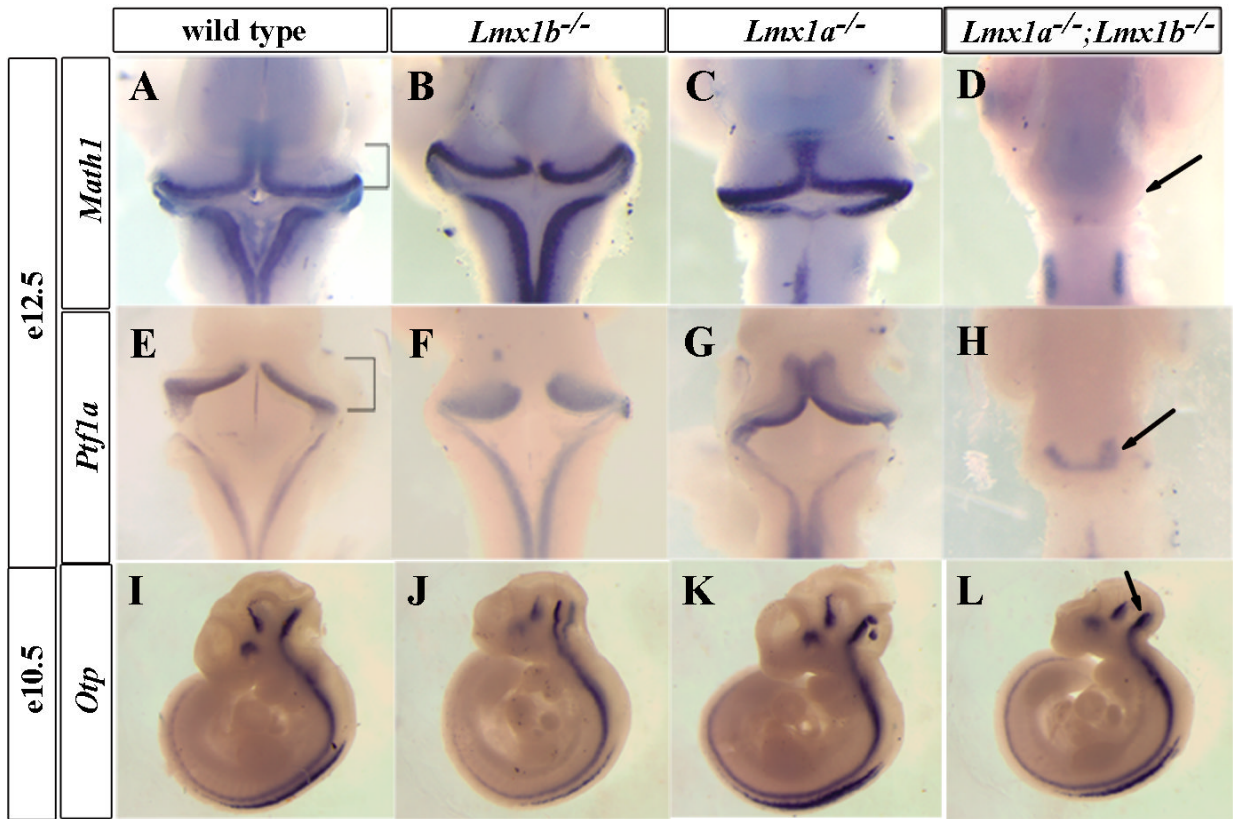
**Figure 1. Expression of *Lmx1a* and *Lmx1b* in rh1 roof plate and its derivative, the choroid plexus epithelium**

(A-D) Whole mount *in situ* hybridization using probes for *Lmx1a* and *Lmx1b*. (A and B) Dorsal views of E8.5 embryos. *Lmx1a* and *Lmx1b* were expressed in roof plate progenitor cells at the lateral edges of the neural tube (arrowheads). *Lmx1b* was also expressed across the isthmus organizer (IsO) at the mid/hindbrain junction (arrow in (B)). (C and D) Lateral view of E10.5 embryos. Brackets indicate rhombomere 1 (rh1). (C) *Lmx1a* was expressed in the roof plate along the anterior posterior axis of the embryo including rh1 (arrowhead). (D) *Lmx1b* was expressed in the anterior roof plate (arrowhead) and in the IsO (arrow). (E) Schematic of dorsal view of rh1 and paramedial sagittal section through rh1 at E10.5. Boxed region is equivalent to sagittal sections in (F-H). (F) Immunohistochemistry at E12.5 showed expression of *Lmx1a* in choroid plexus (CP) and in the rhombic lip (RL) (arrow). (G and H) Arrowhead shows the CP/RL boundary. (G) *Lmx1b* expression was restricted to the CP. (H) Merged image of (F) and (G). (I) Schematic of mid-sagittal section of P6 cerebellum. Boxed region identifies the posterior lobe and the CP shown in the next panel. (J) *Lmx1b* was expressed in the CP but not in the cerebellum. EGL, external granule layer.



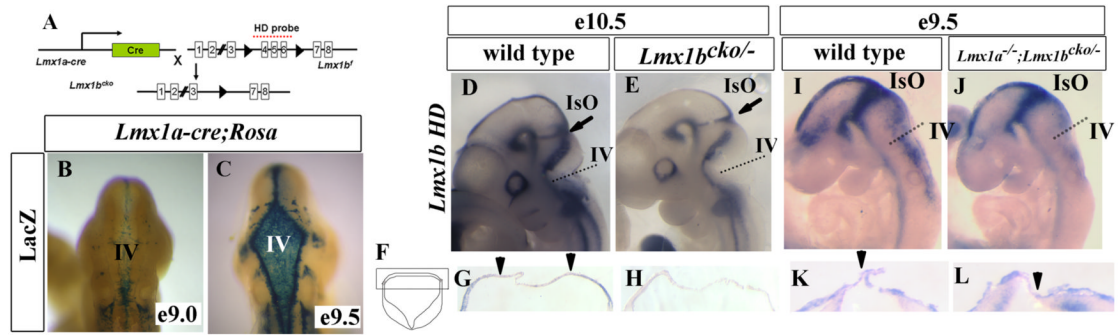
**Figure 2. Development of the hindbrain roof plate is dependent on overlapping functions of *Lmx1a* and *Lmx1b***

(A-H) Dorsal view of whole mount *in situ* hybridization of E10.5 embryos using probes for *Gdf7* and *Wnt1*. The brackets in (A) and (B) indicate the position of rh1 and the future cerebellum. Line in (B) specifies level of transverse section in (I). (C and D) In *Lmx1b*<sup>-/-</sup> embryos, the 4<sup>th</sup> ventricle roof plate was nearly normal despite the loss of the IsO. (E and F) The 4<sup>th</sup> ventricle roof plate was reduced in *Lmx1a*<sup>-/-</sup> embryos. (G and H) *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos had little or no residual 4<sup>th</sup> ventricle roof plate. Line in (H) indicates the transverse section in (K). (I) Schematic of a transverse section of the 4<sup>th</sup> ventricle. Boxed region indicates equivalent region in (J) and (K). (J) In wild-type embryos, the roof plate covered the 4<sup>th</sup> ventricle. (K) This region was lost in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos, although residual, lateral non-roof plate *Wnt1* expression remained. IV; 4<sup>th</sup> ventricle.



**Figure 3. *Math1*+ rhombic lip cells are lost in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos**

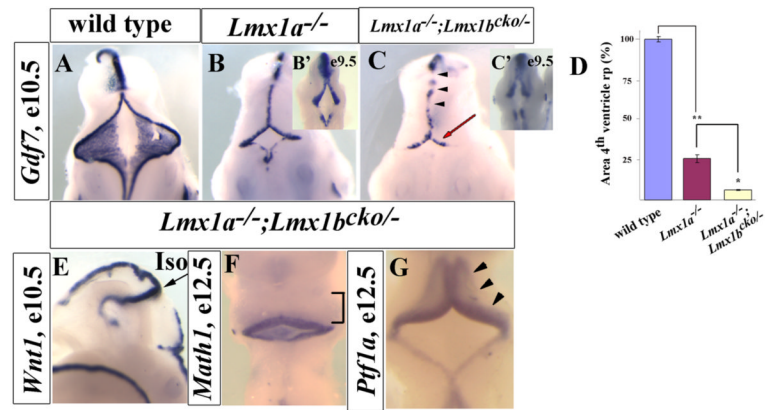
(A-H) Dorsal view of whole mount *in situ* hybridization of sub-dissected E12.5 brains using probes for *Math1* (A-D) and *Ptf1a* (E-H). Brackets in (A) and (E) indicate the developing cerebellum. (A-C) *Math1* expression, induced by roof plate signaling, was retained in *Lmx1b*<sup>-/-</sup> (B), and *Lmx1a*<sup>-/-</sup> (C) embryos. However, *Math1* expression was completely lost in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> embryos (arrow in (D)) confirming complete loss of roof plate function in rh1. (E-H) *Ptf1a* expression is independent of roof plate signaling, and was present in all genotypes, although the domain of expression was reduced in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> mutants (arrow in (H)). (I-L) *Otp* expression was not disturbed in *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>-/-</sup> mutants (arrow in (L)) compared to the wild-type (I) or single mutant embryos (J and K), indicating that alar/basal boundary is not perturbed.



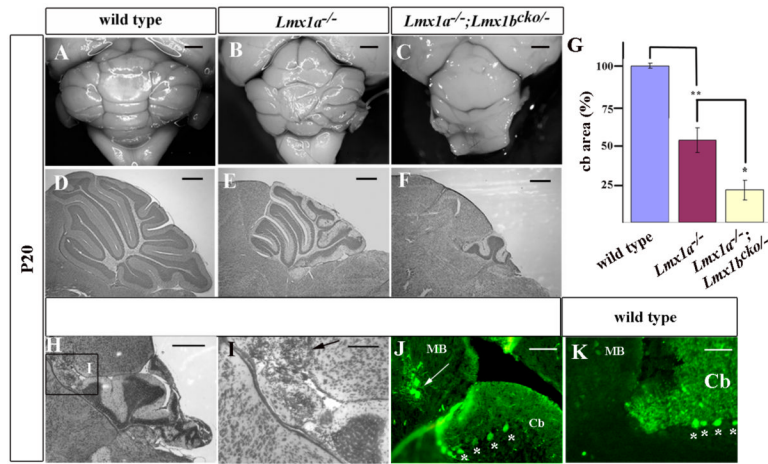
**Figure 4. A conditional knockout strategy to delete *Lmx1b* only in the roof plate where it is co-expressed with *Lmx1a***

(A) Schematic diagram demonstrating the *Lmx1b* conditional knockout strategy. *Lmx1a-cre* transgenic mice were bred to mice carrying a floxed allele of *Lmx1b* (*Lmx1b<sup>f</sup>*), leading to excision of *Lmx1b<sup>f</sup>* only within the *Lmx1a* expression domain in the roof plate. (B and C)  $\beta$ -gal activity in the progeny of *Lmx1a-cre* mice mated to ROSA26 floxed LacZ reporter mice. (B) Only a few LacZ positive cells are visible in the 4<sup>th</sup> ventricle at E9.0. (C) LacZ expression is clearly visible at the 4<sup>th</sup> ventricle at E9.5.

(D-L) *In situ* hybridization using the *Lmx1b* homeodomain (HD) probe indicated in (A). (D and E) Whole mount at E10.5 of wild-type (D) and *Lmx1b<sup>cko/-</sup>* embryos (E). *Lmx1b* HD expression was reduced in the 4<sup>th</sup> ventricle roof plate but remained intact in the IsO in *Lmx1b<sup>cko/-</sup>* embryos. (F) Schematic of a transverse section of the 4<sup>th</sup> ventricle at E10.5. Boxed region indicates equivalent region in (G-H and K-L). (G and H) Transverse sections at E10.5 showed that *Lmx1b* HD expression is present in the wild-type (G) but lost in *Lmx1b<sup>cko/-</sup>* embryos (H). Arrowheads indicate *Lmx1b* HD expression in the wild-type. (I and J) Whole mount at E9.5 showed that *Lmx1b* HD is expressed in both the wild-type (I) and *Lmx1a<sup>-/-</sup>; Lmx1b<sup>cko/-</sup>* embryos (J). (K and L) Transverse sections through the 4<sup>th</sup> ventricle at E9.5 showed that *Lmx1b* HD expression is present in both the wild-type (K) and *Lmx1a<sup>-/-</sup>; Lmx1b<sup>cko/-</sup>* embryos (L). Arrowheads indicate *Lmx1b* HD expression.

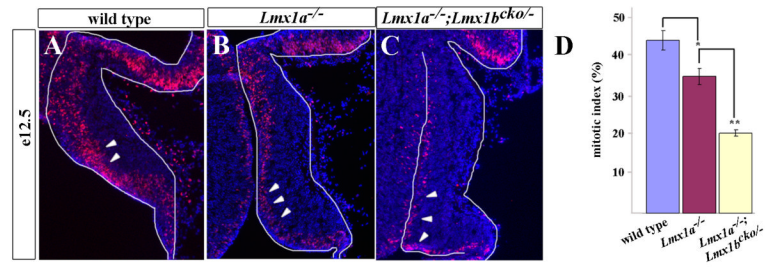


**Figure 5. An intermediate roof plate phenotype is observed in conditional double knockout embryos** (A-C) Dorsal view of E10.5 embryos hybridized with a *Gdf7* *in situ* probe. (B) *Lmx1a*<sup>-/-</sup> embryos had a small 4<sup>th</sup> ventricle roof plate compared to the wild-type. (C) *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> retained the roof plate (unlike the double null embryos) but developed an even smaller 4<sup>th</sup> ventricle roof plate compared to *Lmx1a*<sup>-/-</sup> embryos (red arrow). In addition, *Gdf7* expression was patchy (arrowheads). Insets in panels B and C show roof plate at E9.5 in *Lmx1a*<sup>-/-</sup> embryos (B') and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos (C'). (D) Quantification of 4<sup>th</sup> ventricle roof plate area in wt, *Lmx1a*<sup>-/-</sup>, and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos at E10.5 (n=3). Y-axis is normalized to wt. \* indicates p<0.01; \*\* indicates p<0.001. Bars indicate standard error (se). (E) Lateral view of E10.5 embryo stained with *Wnt1* *in situ* probe. Iso is normally present in *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos. (F and G) Dorsal view of E12.5 *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos stained with *Math1* (F) and *Ptf1a* (G) *in situ* probes. Variability of these markers was not detected, despite reduction in size of the 4<sup>th</sup> ventricle roof plate.



**Figure 6. Cerebellar morphogenesis is dependent on *Lmx1* dependent roof plate signaling**  
 (A-C) Whole mount and (D-F) nissl stained mid-sagittal sections of P20 cerebella demonstrating that loss of both *Lmx1b* and *Lmx1a* had significant effects on cerebellar development. The cerebella of *Lmx1a*<sup>-/-</sup> mice were small, but those of *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> mice were even smaller. (G) Quantification of mid-sagittal sections of P20 cerebella (n=4). Area of wild-type is indicated as 100%. \* indicates p<0.01; \*\* indicates p<0.001. Bars indicate standard error. (H-J, L) Ectopic cerebellar cells were observed in n=2/8 *Lmx1a*<sup>-/-</sup>;*Lmx1b*<sup>cko/-</sup> cerebella in the midbrain are evident in nissl stained sections. (I) Higher magnification of the boxed region in (H) showing ectopic cerebellar cells in the midbrain. (J) White arrows indicate calbindin-positive Purkinje cells present in the midbrain (MB) with cerebellar (Cb) Purkinje cells are indicated by \*, in similar sections. (K) An equivalent wild-type calbindin stained section. Scale bars are (A-C) 1 mm, (D-F) 500 μm, (H) 500 μm, (I-K) 250 μm.





**Figure 7. Reduced proliferation in the cerebellar anlage correlates with reduced roof plate size** (A-C) Midsagittal sections of BrdU (red) and DAPI (blue) stained wild-type (A), *Lmx1a*<sup>-/-</sup> (B), and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> (C) cerebella anlage at E12.5. Arrowheads show BrdU staining in the ventricular zone. Proliferation was reduced in *Lmx1a*<sup>-/-</sup> and *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos.

(D) Mitotic index of E12.5 ventricular zone (BrdU/DAPI). *Lmx1a*<sup>-/-</sup>; *Lmx1b*<sup>cko/-</sup> embryos showed significant reduction compared to *Lmx1a*<sup>-/-</sup> embryos, which was reduced compared to wild-type embryos. \* indicates  $p < 0.01$ ; \*\* indicates  $p < 0.001$ . Bars indicate standard error.