

# The roof plate regulates cerebellar cell-type specification and proliferation

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During embryogenesis, the isthmus organizer, a well-described signaling center at the junction of the mid-hindbrain, establishes the cerebellar territory along the anterior/posterior axis of the neural tube. Mechanisms specifying distinct populations within the early cerebellar anlage are less defined. Using a newly developed gene expression map of the early cerebellar anlage, we demonstrate that secreted signals from the rhombomere 1 roof plate are both necessary and sufficient for specification of the adjacent cerebellar rhombic lip and its derivative fates. Surprisingly, we show that the roof plate is not absolutely required for initial specification of more distal cerebellar cell fates, but rather regulates progenitor proliferation and cell position within the cerebellar anlage. Thus, in addition to the isthmus, the roof plate represents an important signaling center controlling multiple aspects of cerebellar patterning.

**KEY WORDS:** Roof plate, Dorsal midline, Signaling center, Cerebellum, Isthmic organizer, *Lmx1a*, *Bmp*, Neuronal specification, Proliferation, Mouse

## INTRODUCTION

The cerebellum is the primary center of motor coordination and is essential for cognitive processing and sensory discrimination (Schmahmann, 2004). During embryonic development it arises from dorsal rhombomere 1 (r1), adjacent to the fourth ventricle (Chizhikov and Millen, 2003). Normal cerebellar development depends on formation and function of the isthmus organizer (IsO), a signaling center defining the cerebellar territory along the anterior–posterior axis of the central nervous system (CNS) (Liu and Joyner, 2001; Wang and Zoghbi, 2001; Wurst and Bally-Cuif, 2001). As development proceeds, the cerebellar anlage gives rise to several classes of neurons. Fate mapping studies have shown that Purkinje cells and other GABAergic neurons, including a subpopulation of deep cerebellar nuclei (DCN) neurons, basket, stellate and golgi neurons, all arise from the ventricular zone in overlapping waves commencing at embryonic day 10.5–11.5 (E10.5–E11.5) in the mouse (Altman and Bayer, 1997; Goldowitz and Hamre, 1998; Hoshino et al., 2005). The rostral rhombic lip is another germinal matrix producing cerebellar progenitors and represents the dorsal-most neuroectoderm of r1. It is defined by expression of the transcription factor *Math1* (*Atoh1* – Mouse Genome Informatics) (Wang et al., 2005; Machold and Fishell, 2005) (reviewed by Wingate, 2005). *Math1*<sup>+</sup> progenitors give rise to superficially migrating populations of pontomesencephalic neurons, large neurons of the DCN, and cerebellar granule cells in separate birth cohorts (Wang et al., 2005; Machold and Fishell, 2005; Wingate, 2005). Very few cell-type-specific markers within the early cerebellar anlage are currently available, and little is understood regarding the mechanisms driving early cerebellar anlage patterning.

In many CNS regions, the specification of neural types is controlled by secreted signals derived from local signaling centers (Lumsden and Krumlauf, 1996; Briscoe and Ericson, 2001). This is best defined in the developing spinal cord where the neural tube is initially subdivided into gene-expression domains along the dorsal–ventral axis. Each domain gives rise to distinct subclasses of neurons. In the developing spinal cord, the roof plate, a specialized group of cells, differentiates shortly after neural tube closure to form a morphologically distinct narrow strip of cells along the dorsal midline. Spinal cord roof plate cells act as a signaling center producing *Gdf7* and other *Bmp*-related molecules and *Wnt* molecules to non-autonomously control specification of numerous classes of adjacent dorsal interneurons (Lee and Jessell, 1999; Helms and Johnson, 2003; Chizhikov and Millen, 2004b; Chizhikov and Millen, 2005). Intriguingly, many molecules expressed in the spinal cord roof plate are also expressed in dorsal r1, raising the possibility that r1 roof plate acts as a signaling center patterning the adjacent cerebellar anlage and controlling the development of cerebellar cell types.

To address this hypothesis, we first defined several cellular populations of the early cerebellar anlage through gene expression and fate map studies. Using loss- and gain-of-function analysis, we conclude that specification of the cerebellar rhombic lip and its derivative fates is entirely dependent on r1 roof plate. In contrast, roof plate signaling is not absolutely required for specification of more distal cerebellar anlage populations. Rather, roof plate is required for normal positioning of these cells and regulates their numbers by controlling proliferation of the entire cerebellar anlage. Finally, our data indicate that a *Bmp/Lmx1a* pathway is required for r1 roof plate development and that *Bmps* are important components of its signaling.

## MATERIALS AND METHODS

### Mice

Mouse lines used were *Lmx1a*<sup>Δr-J</sup> (Lyons et al., 1996) (Jackson Laboratories); *Math1*<sup>lacZ</sup> (Ben-Arie et al., 2000), *Gdf7-DTA* (Lee et al., 2000) with *ActB-Cre* (Lewandowski et al., 1997) (Jackson Laboratories) for roof plate ablation; *r.p.Lmx1a-Cre* (A.G.L., R. Roberts and K.J.M., unpublished) or *Gdf7-Cre*

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(Lee et al., 2000) with Rosa26 conditional lacZ reporter (129S-Gt(ROSA)26Sortm1Sor/J; Jackson Laboratories) (Soriano, 1999) for *Gdf7* and *Lmx1a* fate mapping studies. Genotyping was previously described (Ben-Arie et al., 2000; Lee et al., 2000; Millonig et al., 2000; Monuki et al., 2001). For transgenic mice, full-length mouse *Lmx1a* cDNA was cloned into pNERV (Panchision et al., 2001) and mice generated by the University of Chicago Transgenic Mouse Facility. Founder embryos were collected at E12.5 and E16.5, and genotyped by PCR (primers are available upon request).

### Tissue analysis

Immunohistochemistry was performed as previously described (Chizhikov and Millen, 2004a), using the following primary antibodies: rabbit anti-Lmx1a (M. German, unpublished), anti-Lhx2/9 (Lee et al., 1998), anti-Ptf1a (H. Edlund, unpublished), mouse anti-Math1, anti-Lhx1/5, anti-Msx1/2 (DSHB, The University of Iowa, Department of Biological Sciences) and rat anti- $\beta$ -galactosidase (T. Glaser, unpublished) together with secondary species appropriate antibodies (Jackson Immunological). *In situ* hybridization was performed as previously described (Chizhikov and Millen, 2004a) using mouse *Lmx1a*, *Gdf7*, *Wnt1*, *Fgf8*, *Math1*, *CyclinD2*, *Ptf1a* and *Lhx5* probes. X-Gal staining, histology, TUNEL assays and BrdU labeling were performed as previously described (Currell et al., 2005; Panchision et al., 2001).

### Explants

E8.5 dorsal neural plate and E8.75-E9.0 intermediate neural tube explants were isolated as described by Alder et al. (1999). Stage 12-15 chick r1 roof plate was isolated as described by Liem et al. (Liem et al., 1997) and co-cultured directly attached to intermediate neural explants. Explants were cultured (Alder et al., 1999) with or without noggin and follistatin (R&D Systems). *Gdf7* expression was assayed by semi-quantitative RT-PCR as previously described (Chizhikov and Millen, 2004c).

### Measurements and statistical analysis

Serial 10  $\mu$ m transverse or sagittal sections covering the entire mid-hindbrain region were collected. Appropriate sections of r1 were identified based on morphology and En1/2 staining. Quantitative data obtained from analysis of paramedial sagittal (*Lmx1a* transgenic and *dreher* embryos) and transverse (roof plate ablated embryos) sections are presented. Quantitative data are expressed as the mean  $\pm$  s.e.m. Statistical significance was determined by *t*-test, \**P* < 0.01.

## RESULTS

### Rhombomere 1 roof plate has distinct anterior and posterior domains and is defined by *Lmx1a* and *Gdf7* expression

The morphology of roof plate varies along the anterior-posterior axis of r1 (Fig. 1A). Therefore we used gene expression to unambiguously define the r1 roof plate. At E10.5, immediately posterior to the isthmus, low levels of *Lmx1a* and *Gdf7* expression are restricted to a narrow medial domain in the anterior region of r1, which does not express *Wnt1* (Fig. 1B-D,J). Posteriorly, *Lmx1a* is expressed both in an expanded medial single cell layer and in adjacent cells (Fig. 1B,E,J,K). Expression of *Gdf7* and *Wnt1* in posterior r1 is illustrated in Fig. 1C,D and summarized in Fig. 1J,K. Lack of *Gdf7* and *Wnt1* antibodies prevented a direct comparison of these genes with *Lmx1a* at single-cell resolution level. However, analysis of *Gdf7*-Cre;*Rosa26* embryos, which express *LacZ* in all *Gdf7*-expressing cells and progeny (Fig. 3A), revealed complete overlap between *LacZ* and *Lmx1a* expression in both anterior and posterior r1 at E10.5 (Fig. 1F and data not shown). Based on double antibody staining, there was no significant overlap between *Math1*, a marker of the rhombic lip, and *Lmx1a* at E10.5 (Fig. 1G). These data indicate that *Lmx1a* and *Gdf7* define the same population of cells, which is distinct from other dorsal populations within both anterior and posterior r1 at E10.5. We define these cells as the r1 roof plate (Fig. 1J,K). At E10.5, the *Lmx1a*<sup>+/Gdf7</sup> edge region of the posterior r1 roof plate is mitotically

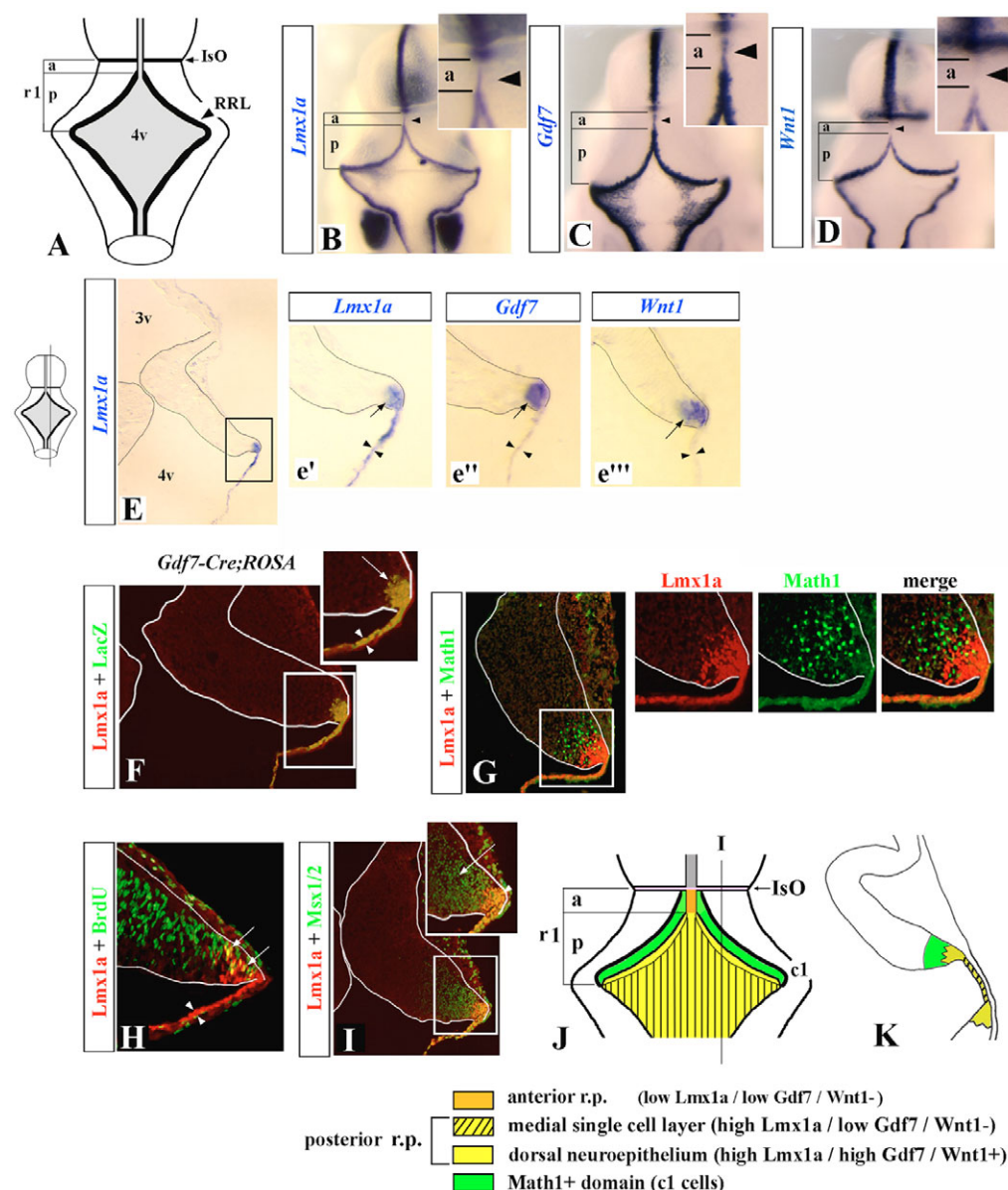
active, as is the entire cerebellar anlage (Fig. 1H). Consistent with *Bmp* r1 roof plate expression, all *Lmx1a*<sup>+</sup> roof plate cells and the adjacent neuroepithelial cells, express *Msx1/2*, downstream markers of *Bmp* signaling (Fig. 1I).

### Transcription factors define distinct cellular populations within the early cerebellar anlage

By analyzing the expression of transcription factors which distinguish neuronal populations in the spinal cord, we defined four cellular populations (denoted c1-c4) within the E12.5 cerebellar anlage. c1 cells arise adjacent to the r1 roof plate and express *Math1* (Ben-Arie et al., 1996; Alder et al., 1999). Similar to E10.5 embryos, only very minimal overlap was detected between *Lmx1a* and *Math1* at E12.5 (Fig. 2A) suggesting that *Math1*<sup>+</sup> cells remain distinct from *Lmx1a*<sup>+</sup> roof plate cells (Fig. 2G,H). Beginning from E10.0, c1 cells and their progeny migrate rostrally and laterally to form the rostral rhombic lip migratory stream (RLS) and immediately downregulate *Math1* as they exit the rhombic lip territory (Wang et al., 2005). To develop early markers of the RLS we used *Math1*<sup>lacZ/+</sup> embryos, in which all RLS cells are transiently labeled by cytoplasmic *LacZ* expression. Co-labeling revealed that at E12.5, nuclear *Lhx2/9* (*Lh2A/B*) expression was initiated immediately as c1 cells became engaged into the RLS and was detected in all RLS cells, including those in the cerebellar anlage and pons (Fig. 2B,C). *Lhx2/9* is therefore an early marker of the RLS.

c2 and c4 cells express *Lhx1/5*, while c3 cells express *Lmx1a* (Fig. 2A,D,E). c2 cells are located directly adjacent to the *Math1*<sup>+</sup> c1 cells and can be detected along the entire length of r1, except the most anterior domain (Fig. 2G). At E13.5, *Lhx1/5*<sup>+</sup> c2 cells initiate expression of the Purkinje cell-specific marker calbindin as they migrate toward the cerebellar surface (see Fig. S1A in the supplementary material). At later embryonic stages and in the adult cerebellum, these cells coexpress *Lhx1/5* and calbindin, and acquire typical morphology of Purkinje cells (see Fig. S1B in the supplementary material). In the adult, *Lhx1/5* is also expressed in GABA<sup>+</sup> stellate, basket and Golgi cells and in small DCN GABAergic neurons (see Fig. S1B in the supplementary material), all of which originate from *Ptf1a*-expressing progenitors (Hoshino et al., 2005). Comparison of *Ptf1a* and *Lhx1/5* expression at E12.5 revealed that the *Ptf1a* expression precisely abuts c2 *Lhx1/5* expression. Furthermore, a small number of cells coexpress both these proteins (Fig. 2D), strongly suggesting that *Ptf1a*<sup>+</sup> cells are progenitors of *Lhx1/5*<sup>+</sup> c2 cells. Therefore, we define *Ptf1a*<sup>+</sup> cells as pc2 (progenitors of c2) cells. Similar to c2 cells, c4 cells also express *Lhx1/5*, but are located at a more ventral position within the E12.5 cerebellar anlage (Fig. 2A,D). The fate of c4 cells is unknown.

The c3 population consists of *Lmx1a*<sup>+</sup>/*Msx1/2*<sup>-</sup> cells (Fig. 2E). These are located between c2 and c4 cells (Fig. 2A) and are found along the entire r1 except the most anterior domain (Fig. 2G). c3 cells initiate *Lmx1a* expression around E11.5-12.5 and represent the only group of *Lmx1a*<sup>+</sup> cells within the early cerebellar anlage (Fig. 2A,E). The location of c3 cells at E12.5 resembles the nuclear transitory zone, a transient differentiation zone of DCN neurons (Altman and Bayer, 1985). At E17.5-E18.5, c3 cells become segregated into several clusters found at the base of the cerebellar anlage (see Fig. S1C in the supplementary material) corresponding to the positions of the nascent DCN. Furthermore, at E14.5-E17.5 some of *Lmx1a*<sup>+</sup> cells express calretinin, a marker for a large population of differentiating DCN neurons (Jacobowitz and Abbott, 1998) (see Fig. S1D in the supplementary material). This suggests that c3 cells give rise to DCN neurons. However, *Lmx1a* is localized to cells with large nuclei which do not express GABA. This is in



**Fig. 1. Cellular populations in dorsal rhombomere 1 at E10.5.** (A) Dorsal view schematic of r1. Anterior (a) and posterior (p) domains of r1 are indicated. 4v, 4th ventricle, IsO, isthmus, RRL, rostral rhombic lip. (B-D) Whole-mount in situ staining of E10.5 r1 demonstrating anterior (a) and posterior (p) roof plate domains. Arrowhead indicates reduced expression of *Lmx1a* and *Gdf7* and no *Wnt1* expression in the anterior domain. (E-I) In situ and immunostained paramedial sagittal sections through r1 in wild-type (E, G-I) and *Gdf7*-*Cre*;ROSA (F) E10.5 mice. Markers are indicated. Arrowheads in e'-e''' point to the medial single cell layer. Arrows point to dorsal neuroepithelium, which expresses *Lmx1a*, *Gdf7* and *Wnt1*. (F) Arrowheads and arrow point to the medial single cell layer and dorsal neuroepithelium, respectively. (H) Arrows point to BrdU\*/*Lmx1a*<sup>+</sup> double-labeled cells. Arrowheads point to the medial single cell layer. (I) Arrowhead indicates *Lmx1a*<sup>+</sup>/*Msx1*/2<sup>+</sup> region and the arrow indicates the *Msx1*/2<sup>+</sup> region outside of this domain (J,K) Summary diagrams of gene expression domains, with provided key. Roof plate is defined as the *Lmx1a*<sup>+</sup>/*Gdf7*<sup>+</sup> region in both anterior and posterior r1.

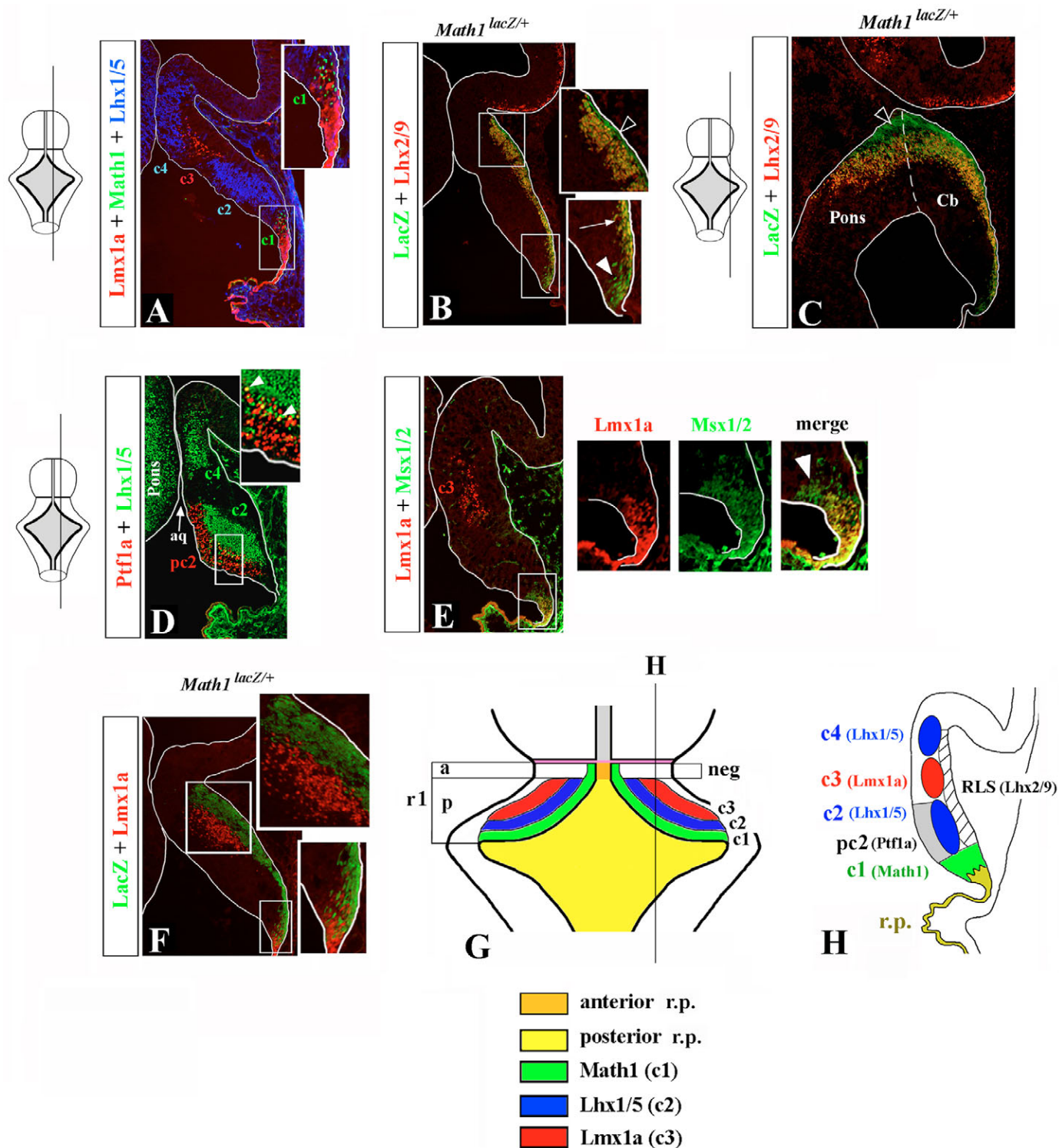
contrast to *Lhx1/5* which is expressed in cells with small nuclei (data not shown). Thus, c2 and c3 cells give rise to distinct cellular populations. Since RLS cells also give rise to some large DCN neurons, we assessed *Lmx1a* expression in *Math1*<sup>LacZ/+</sup> embryos. We found that c3 cells do not originate from the RLS (Fig. 2F) suggesting that c3 cells give rise to a previously unrecognized population of DCN cells.

Our data argue that by E12.5, the early cerebellar anlage has already been subdivided into discrete gene-expression domains, each of which gives rise to distinct cerebellar populations. These markers can now be used to assess mechanisms driving early cerebellar anlage patterning.

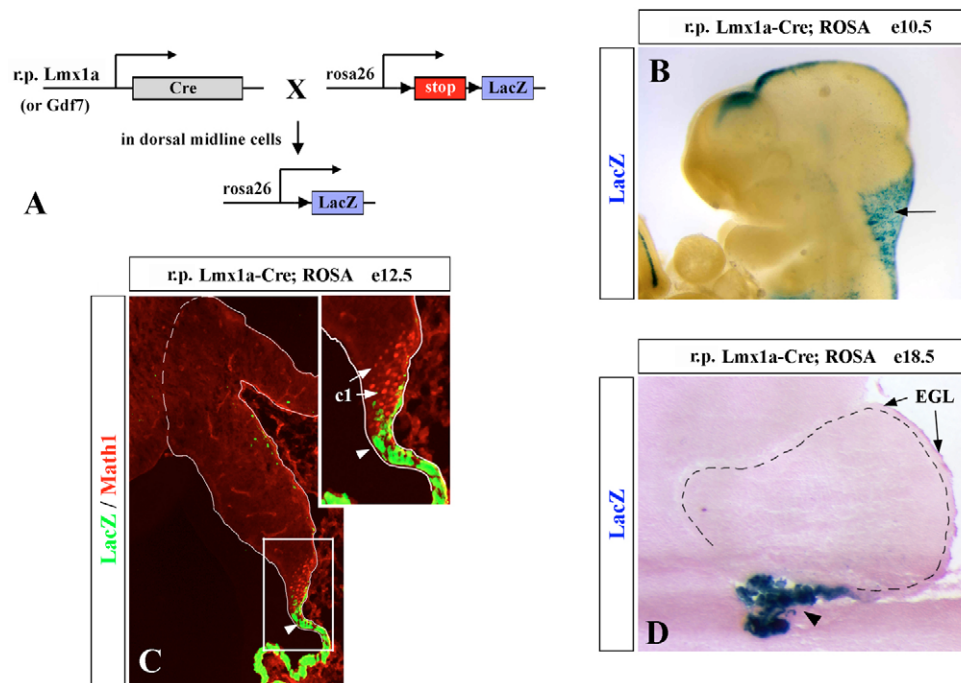
### Rhombomere 1 roof plate gives rise to choroid plexus and does not significantly contribute to the developing cerebellum

Lineage tracing studies in the mouse have revealed that the roof plate contributes to several neuronal populations in both the developing spinal cord and the forebrain (Lee et al., 2000;

Monuki et al., 2001). In r1, *Lmx1a*<sup>+</sup>/*Gdf7*<sup>+</sup> roof plate cells are located directly adjacent to *Math1*<sup>+</sup> c1 cells suggesting that some rhombic lip cells or other cerebellar cells may be clonally related to roof plate. To address this possibility we performed genetic fate mapping studies. We used mice expressing Cre recombinase under the control of the *Lmx1a* promoter (*r.p.Lmx1a-Cre*; A.G.L., R. Roberts and K.J.M., unpublished), which specifically drives expression of an endogenous gene to the roof plate at early developmental stages, or the *Gdf7* promoter (*Gdf7-Cre*) (Lee et al., 2000). These mice were crossed with ROSA26 cre reporter mice (Soriano, 1999) to permanently label roof plate derivatives (Fig. 3A,B). In E12.5 *r.p.Lmx1a-Cre*;ROSA26 embryos, LacZ staining was detected in the choroid plexus, with no detectable overlap between LacZ and c1 marker *Math1* (Fig. 3C). At E18.5, very few LacZ<sup>+</sup> cells were detected within the cerebellum (Fig. 3D and data not shown). This suggests therefore, that the roof plate contributes cells to the choroid plexus and is not a significant source of cerebellar cells.



**Fig. 2. Cellular populations within the E12.5 cerebellar anlage.** (A,B,D-F) Paramedial sagittal and (C) lateral sagittal immunostained sections through dorsal r1 at E12.5 in wild-type (A,D,E) and *Math1*<sup>lacZ/+</sup> (B,C,F) embryos. Markers are indicated. Solid arrowhead in B points to LacZ<sup>+</sup> cells in the rhombic lip many of which are Lhx2/9 negative. Arrow points to LacZ<sup>+</sup>/Lhx2/9<sup>+</sup> cells engaged in the RLS. The open arrowheads in B and C indicate LacZ<sup>+</sup> fibers on the dorsal surface, which belong to LacZ<sup>+</sup>/Lhx2/9<sup>+</sup> cells. Arrowheads in D inset indicate small numbers of Ptf1a<sup>+</sup>/Lhx1/5<sup>+</sup> double-labeled nuclei. Laterally, the junction (broken line in C) between the pons and cerebellum (Cb) is difficult to distinguish, since the aqueduct (aq in D) no longer separates them. Arrowhead in merge panel E indicates Lmx1a<sup>+</sup>/Msx1/2<sup>+</sup> cells. (G,H) Schematic of the molecular map of roof plate r1 populations in whole-mount (G) and paramedial sagittal section (H). c1 cells extend to the isthmus adjacent to the anterior roof plate. E12.5 cerebellar anlage populations are excluded from this region, defining a negative (neg) zone of gene expression.



**Fig. 3. Roof plate cells do not significantly contribute to the cerebellar anlage.** (A) Schematic of the genetic strategy to permanently label progeny of roof plate cells. Black triangles indicate loxP sites. (B) Side view of X-Gal-stained E10.5 *r.p.Lmx1a-cre;ROSA* embryo. LacZ expression (arrow) recapitulates dorsal *Lmx1a* expression at E10.5. (C) Sagittal section of the E12.5 anlage of immunostained *r.p.Lmx1a-cre;ROSA* embryo. Arrowhead points to LacZ<sup>+</sup> cells. Arrows point to Math1<sup>+</sup> c1 cells (D) Sagittal section of X-Gal-stained E18.5 *r.p.Lmx1a-cre;ROSA* embryo. External granule cell layer (EGL) is marked by broken line and arrows. Arrowhead indicates X-Gal<sup>+</sup> choroid plexus.

### A Bmp/Lmx1a pathway is involved in rhombomere 1 roof plate formation and can repattern the early cerebellar anlage

In the developing spinal cord, overexpression of *Lmx1a* induces ectopic roof plate, which, in turn, repatterns the caudal neural tube (Chizhikov and Millen, 2004a). To test if *Lmx1a* has comparable activity in r1, we generated *Lmx1a* transgenic mouse embryos overexpressing *Lmx1a* under control of the nestin promoter, driving expression to the neuroepithelium from at least E9.5 (Panchision et al., 2001).

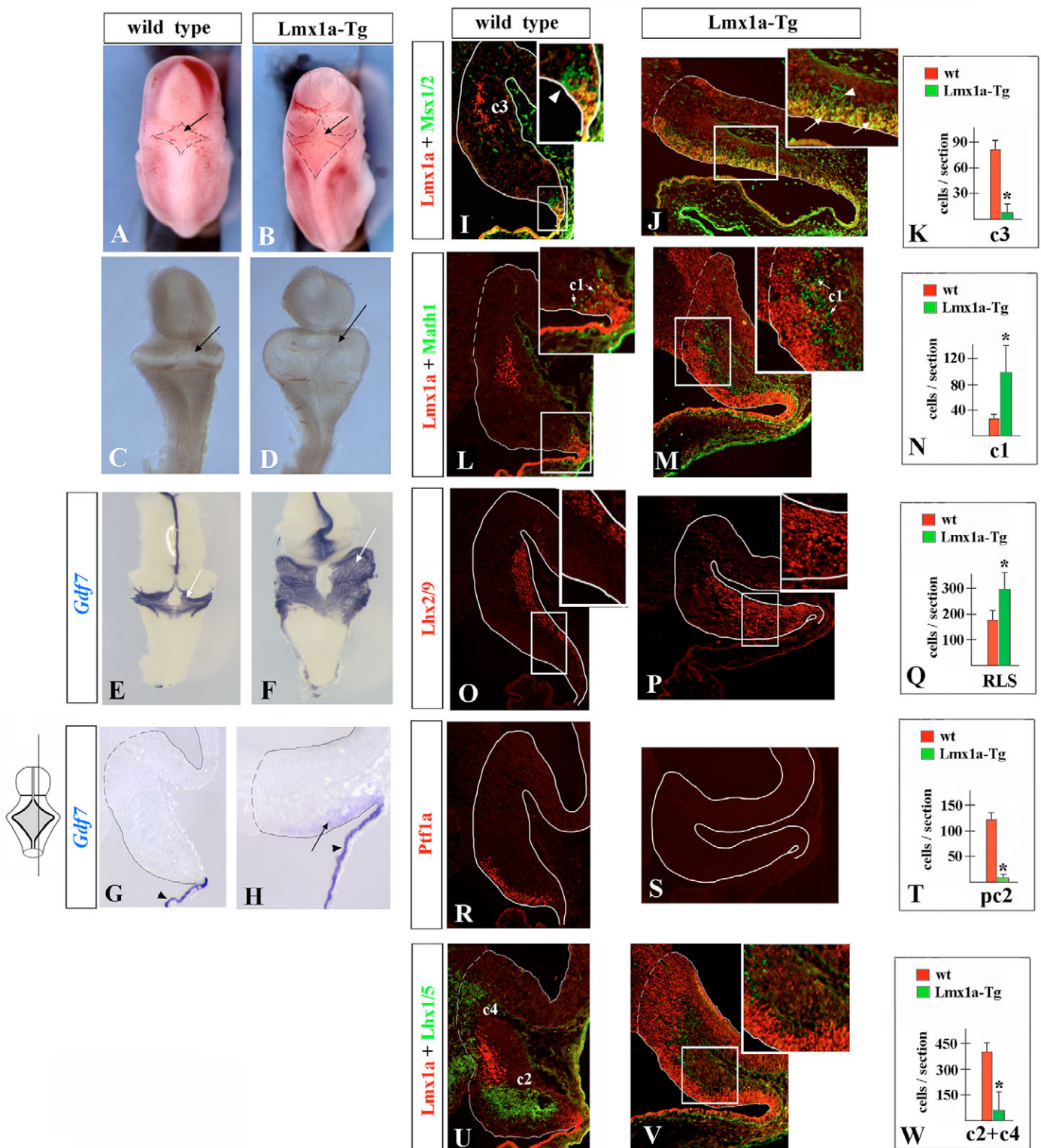
At E12.5, approximately 50% (6/11) of the transgenic embryos strongly expressing exogenous *Lmx1a* had a giant medial single cell layer in r1 (Fig. 4A-F). *Gdf7* was broadly activated in the cerebellar anlage in most (3/4) transgenic embryos investigated (Fig. 4G,H). Markers of Bmp activity, *Msx1/2*, were also ectopically activated in most (4/6) *Lmx1a* transgenic embryos, both in cells expressing exogenous *Lmx1a* and in adjacent cells (Fig. 4J,I), revealing an expanded domain of Bmp signaling in *Lmx1a*-transgenic embryos. Thus, *Lmx1a* is sufficient to induce ectopic roof plate in the developing cerebellar anlage.

*Lmx1a* overexpression also induced numerous c1 cells throughout the transgenic cerebellar anlage (4/6 embryos) (Fig. 4L-N). Induction of c1 cells was associated with additional RLS Lhx2/9<sup>+</sup> cells (3/6 embryos), with some appearing at ectopic positions in the ventricular zone (Fig. 4O-Q). At E16.5, *Lmx1a* transgenic embryos (2/3) also had increased numbers of cerebellar granule progenitors in the external granule layer (see Fig. S2A-C in the supplementary material). In contrast, Ptf1a<sup>+</sup> pc2, Lhx1/5<sup>+</sup> c2 and c4 cells, and *Lmx1a*<sup>+</sup>/*Msx1/2*<sup>-</sup> c3 cells were greatly reduced in numbers in most (4/6) *Lmx1a*-transgenic embryos at E12.5 (Fig. 4I-K,R-W). Consistent with this, at E16.5 there was severe reduction or loss of calbindin<sup>+</sup> Purkinje cells and GABAergic DCN neurons and Golgi cells in most (2/3) *Lmx1a* transgenics (see Fig. S2D-G in the supplementary material). These results indicate that *Lmx1a* is sufficient not only for r1 roof plate development, but it can also repattern the developing cerebellar anlage and substantially alter subsequent cerebellar cell fates.

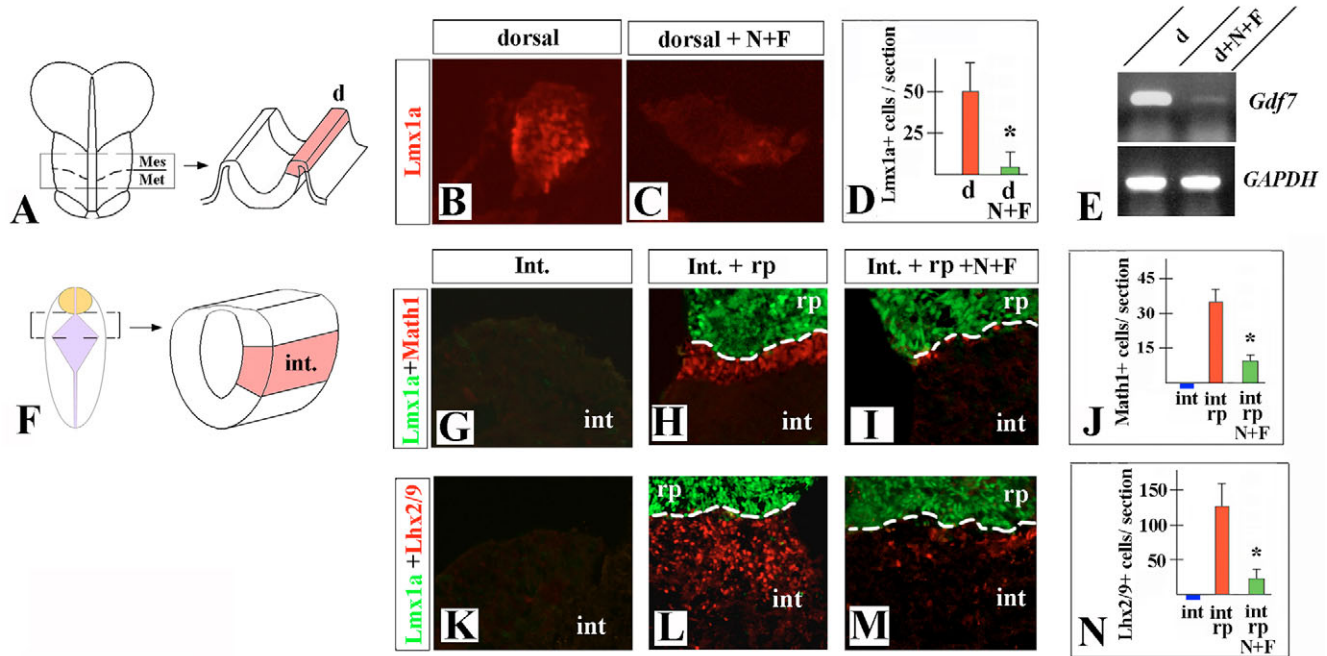
To identify upstream components of the *Lmx1a* signaling pathway we concentrated on Bmps, since *Bmp6* and *Bmp7* are expressed in the rostral epidermal ectoderm at the time of r1 roof plate formation (Furuta et al., 1997; Dudley and Robertson, 1997). We isolated the dorsal-most edges of the r1 neural plate together with adjacent epidermal ectoderm at E8.5, when *Lmx1a* expression is initiated (Fig. 5A). When cultured alone for 24 hours, numerous *Lmx1a*<sup>+</sup> cells and high levels of *Gdf7* expression (Fig. 5B-E) were induced, indicating successful formation of roof plate in vitro. This induction was significantly blocked by the addition of Bmp inhibitors noggin (30 nM) and follistatin (130 nM) (Fig. 5B-E). Therefore, Bmps act upstream of *Lmx1a* in the r1 roof plate-inducing signaling pathway.

### Bmps are necessary mediators of roof plate signaling in the developing cerebellar anlage

Previously, Alder et al. (Alder et al., 1999) determined that Bmps can induce granule cells when added to naïve r1 neural tissue in vitro, suggesting that they may also be mediators of r1 roof plate signaling. To determine if roof plate Bmps are required for its patterning activity, we performed explant experiments. Specifically, we tested whether roof plate-dependent induction of c1 cells was blocked by noggin and follistatin using intermediate explants from E8.75-E9.0 mouse mid/hindbrain neural plate or newly formed neural tube (Fig. 5F). Explants were cultured for 3 days alone or together with stage 12-15 chicken r1 roof plate with or without noggin and follistatin. No Math1<sup>+</sup> c1 or Lhx2/9<sup>+</sup> RLS cells were detected when intermediate explants were cultured alone, but significant numbers of both Math1<sup>+</sup> and Lhx2/9<sup>+</sup> cells were induced when they were co-cultured together with chick r1 roof plate (Fig. 5G-N). Addition of noggin (20 nM) and follistatin (85 nM) did not affect the mature roof plate used in these experiments, as revealed by its normal *Lmx1a* (Fig. 5I,M) and *Gdf7* expression (data not shown), but significantly blocked (by 80-85%) induction of both c1 and Lhx2/9<sup>+</sup> RLS cells (Fig. 5I,M,J,N). Thus we conclude that Bmps in r1 roof plate are required for induction of c1 cells and their derivative RLS cells.



**Fig. 4. Cerebellar anlage abnormalities in mouse embryos with ectopic expression of *Lmx1a*.** (A-F) Dorsal view of E12.5 whole embryos (A, B) and their dissected neural tubes (C-F). Arrows indicate dramatically expanded medial single cell layer in transgenics (B, D, F). (G-W) Paramedial sagittal sections of E12.5 cerebella with genotypes and markers indicated, together with quantification of each cell type. In all panels, insets show a higher magnification of the boxed regions. Roof plate (arrow in H showing *Gdf7* and arrows in J showing *Lmx1a*<sup>+</sup>/*Msx1/2*<sup>+</sup> cells) was expanded to the ventricular surface within the anlage. Arrowheads in I and J point to *Lmx1a*<sup>+</sup>/*Msx1/2*<sup>+</sup> cells located adjacent to *Lmx1a*<sup>+</sup>/*Msx1/2*<sup>+</sup> roof plate cells. (K, N, Q, T, W) Quantification of number of c1, RLS, pc2 and c2-c4 cells in *Lmx1a* transgenic embryos. \* indicates  $P < 0.01$ . Bars indicate s.e.m.



**Fig. 5. Bmps are required for roof plate development and induction of c1 and RLS cells in vitro.** (A-E) Isolation and culturing of e8.5 mid-hindbrain dorsal (d) neural fold explants. Addition of noggin and follistatin (N+F) decreased induction of *Lmx1a* (B-D) and *Gdf7*, as measured by RT-PCR (E). (D) Quantification of numbers of *Lmx1a*<sup>+</sup> cells in dorsal explants cultured with and without Bmp inhibitors. (F-N) Examples of wild-type E8.75-E9.0 intermediate neural tube explants (int) at the level of r1, cultured with and without chick r1 roof plate (r.p.). Addition of noggin and follistatin (N+F) reduces induction of c1 and RLS cells. Intermediate explants and roof plate are labeled and demarked by the broken line. (J,N) Quantification of numbers of *Math1*<sup>+</sup> (J) and *Lhx2/9*<sup>+</sup> (N) cells appeared in wild-type intermediate explants. Blue, intermediate explants cultured alone (int). Red, intermediate explants cultured with chick roof plate (int+rp). Green, intermediate explants cultured with chick roof plate together with noggin and follistatin (int+rp+N+F). \* indicates  $P < 0.01$ . Bars indicate s.e.m.

### Cerebellar anlage abnormalities in *dreher* (*Lmx1a*<sup>-/-</sup>) mouse embryos

To investigate the effects of roof plate disruption on cerebellar anlage development, we first analyzed *dreher* (*Lmx1a*<sup>-/-</sup>) embryos. In the *dreher* mouse, inactivation of *Lmx1a* by point mutation prevents roof plate formation in the developing spinal cord (Millonig et al., 2000; Chizhikov and Millen, 2004a). In *dreher* embryos, r1 roof plate territory is severely reduced in size starting from E9.5, but limited marker analysis suggested that r1 roof plate cells are still present (Millonig et al., 2000). Here we performed more detailed analysis. Our data indicate that the anterior roof plate, which expresses low *Gdf7* levels and does not express *Wnt1*, is expanded at the expense of posterior r1 roof plate in *dreher* embryos (Fig. 6A-D,G-H). *Gdf7* and *Wnt1*, however, are still expressed in the reduced posterior r1 roof plate of E10.5 *dreher* embryos (Fig. 6A-D). These data indicate that although *Lmx1a* is not absolutely required for development of the roof plate in r1, it is critical for a normal relationship between anterior and posterior domains of r1 roof plate. Abnormal patterning of the r1 roof plate did not affect development of the Iso, which was normal in the *dreher* embryos, based both on morphological criteria and retention of isthmus-specific expression of *Wnt1*, *Fgf8*, *Gbx2* and *Otx2* (Fig. 6C,D and data not shown). Thus, loss of *Lmx1a* causes abnormal development of r1 roof plate but does not interrupt global anterior-posterior patterning of the mid/hindbrain territory.

*c1-Math1* expression in the anterior region of *dreher* r1 at both E10.5 and E12.5 was lost, although appropriate numbers of c1 cells were detected in the rest of r1 (Fig. 6E,F,I,J; see Fig. 3E-G in the supplementary material). Thus, *Lmx1a* expression in the roof plate

is specifically required for generation of c1 cells in the anterior domain of r1 even though low levels of *Gdf7* were still expressed in *dreher* anterior r1 roof plate (Fig. 6B). Low levels of *Gdf7* alone, therefore, are not sufficient to induce c1 cells in this specific domain.

We did not detect any defects in expression of *Ptf1a*, *Lhx1/5* or *Lmx1a*, marking the pc2, c2 and c3 cell populations (Fig. 6K-R; see Fig. S3 in the supplementary material) at E12.5 in *dreher* embryos. These data indicate that the relatively mild abnormality in r1 roof plate is not sufficient to grossly disrupt patterning of the E12.5 cerebellar anlage in this mouse model.

The observation that the anterior limit of *Ptf1a*, *Lhx1/5* and *Lmx1a* was unchanged in *dreher* embryos was surprising. In wild-type embryos, these genes are expressed only within the posterior r1 domain and not the anterior 'negative' domain (labeled as 'neg' in Fig. 6K-R). This negative domain normally correlates with the morphological extent of the anterior roof plate (labeled as 'a' in Fig. 6). In *dreher* embryos these two domains no longer coincide (Fig. 6Q,R). Two hypotheses can explain this. First, anterior roof plate is sufficient to support formation of pc2, c2 and c3 cells when expanded into caudal r1. Alternatively, the development of pc2, c2 and c3 cells may be independent of r1 roof plate signals.

### Cerebellar anlage abnormalities following roof plate ablation by *Gdf7*-mediated diphtheria toxin expression

To investigate the effect of complete loss of the roof plate on early cerebellar anlage development, we moved to a genetic ablation mouse model using a mouse line that conditionally expresses the diphtheria toxin A subunit (DTA) from the *Gdf7* locus beginning

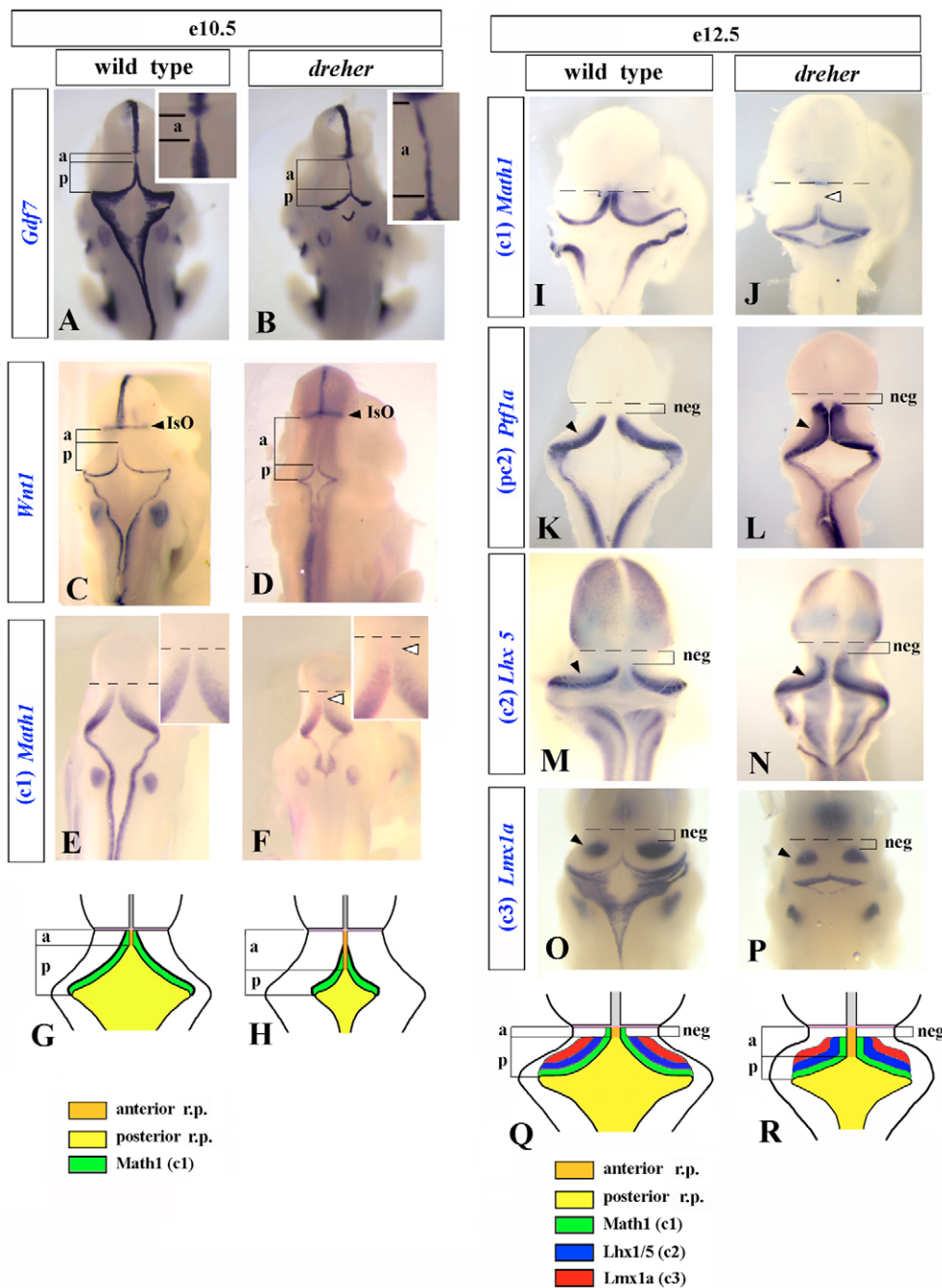
at E9.0 (Fig. 7A) (Lee et al., 2000). Expression from this conditional *Gdf7* allele faithfully recapitulates expression of endogenous *Gdf7* (Lee et al., 2000; Currle et al., 2005) (data not shown).

To ablate *Gdf7*-expressing roof plate cells, we crossed *Gdf7x-Stop-xDTA* mice with *ActB-Cre* mice which ubiquitously express Cre under control of  $\beta$ -actin promoter (Fig. 7A). By E9.5, all *Gdf7x-Stop-xDTA;ActB-Cre* embryos exhibited an open neural tube at the r1 level (e.g. Fig. 7C). We therefore performed most of our analysis in whole-mount and transverse sections.

To confirm specificity and determine timing of r1 roof plate ablation, we performed TUNEL labeling at E8.5-E12.5. At E8.5, no differences in apoptosis or neural plate morphology were detected between wild-type and ablated embryos (data not shown). In E9.5 roof plate-ablated embryos, apoptosis was specifically elevated in

the most dorsal edges of r1, exactly where *Gdf7* is normally expressed (see Fig. S4A,B in the supplementary material). At E10.5-E12.5, no significant differences in apoptosis were detected between wild-type and ablated embryos (see Fig. S4C,D in the supplementary material; data not shown), indicating the temporal and spatial specificity of ablation. Further, no dorsal expression of *Lmx1a*, *Gdf7* or *Wnt1* was detected in the mutant embryos (Fig. 7B-E and data not shown) revealing complete loss of the r1 roof plate cells. Despite the open neural tube, the IsO was developed normally in ablated embryos, as assayed by *Wnt1* and *Fgf8* expression (Fig. 7D,E), indicating that roof plate is not required for maintenance of the IsO.

There was complete loss of *Math1*<sup>+</sup> c1 cells in both E10.5 (Fig. 7F,G) and E12.5 (Fig. 8G,H) roof plate-ablated embryos. Also, no *Lhx2/9*<sup>+</sup> RLS cells were detected in the cerebellar anlage or pons of



**Fig. 6. Roof plate and early cerebellar anlage defects in *dreher* mouse embryos.** (A-F) Dorsal views of whole-mount in situ-stained E10.5 embryos with genotypes and markers as indicated.

In *dreher*, the anterior roof plate (a) is expanded at the expense of the posterior roof plate domain (p). IsO is still maintained (C,D). (E,F) The anterior limit of *Math1* expression is found at the isthmus (broken line) in wild-type embryos. It is positioned more posteriorly in *dreher*. Open arrowheads show *Math1*-negative domain in *dreher* embryos. (I-P) Whole-mount in situ analysis reveals the anterior *Math1* gene expression limit is still abnormal in E12.5 *dreher* embryos. The anterior limits of other genes, however, are normal in *dreher* embryos. Despite morphological changes in the roof plate a normal negative (neg) zone of gene expression is maintained adjacent to the isthmus (broken line in I-P). Open arrow points to *Math1*-negative domain in anterior r1 of *dreher* embryo. Solid arrowheads show pc2, c2 and c3 cells. (G,H,Q,R) Schematic illustrations summarize gene expression changes.

roof plate-ablated embryos at E12.5 (Fig. 8M,N). These data indicate that loss of r1 roof plate prevents formation of both c1 cells and their derivative *Lhx2/9<sup>+</sup>* RLS cells.

At E12.5, pc2, c2 and c3 cells were present in the roof plate-ablated embryos, although in reduced numbers (Fig. 8A-D,G-L,O,P). Thus, roof plate is not absolutely required for specification of pc2, c2 and c3 cells, but does regulate their numbers. Since c3 cells were shifted laterally and dorsally to positions normally occupied by RLS cells (Fig. 8G,H), roof plate is also required for normal positioning of c3 cells within the developing cerebellar anlage.

Reduced numbers of pc2, c2 and c3 cells (Fig. 8I,L) and the overall reduced size of the cerebellar anlage (Fig. 7L) suggested abnormal proliferation in ablated embryos. This was confirmed by BrdU labeling at E10.5 (Fig. 7M,N). The decrease in proliferation is unlikely to be mediated by loss of c1 cells in ablated embryos, since no gross proliferation abnormalities were detected in E10.5 *Math1<sup>LacZ/LacZ</sup>* embryos in which development of c1 cells is severely disrupted (data not shown). Instead, decreased proliferation may be associated with loss of dorsal expression of the well-known mitogen *Wnt1* in roof plate ablated embryos (Fig. 7D,E). In the developing

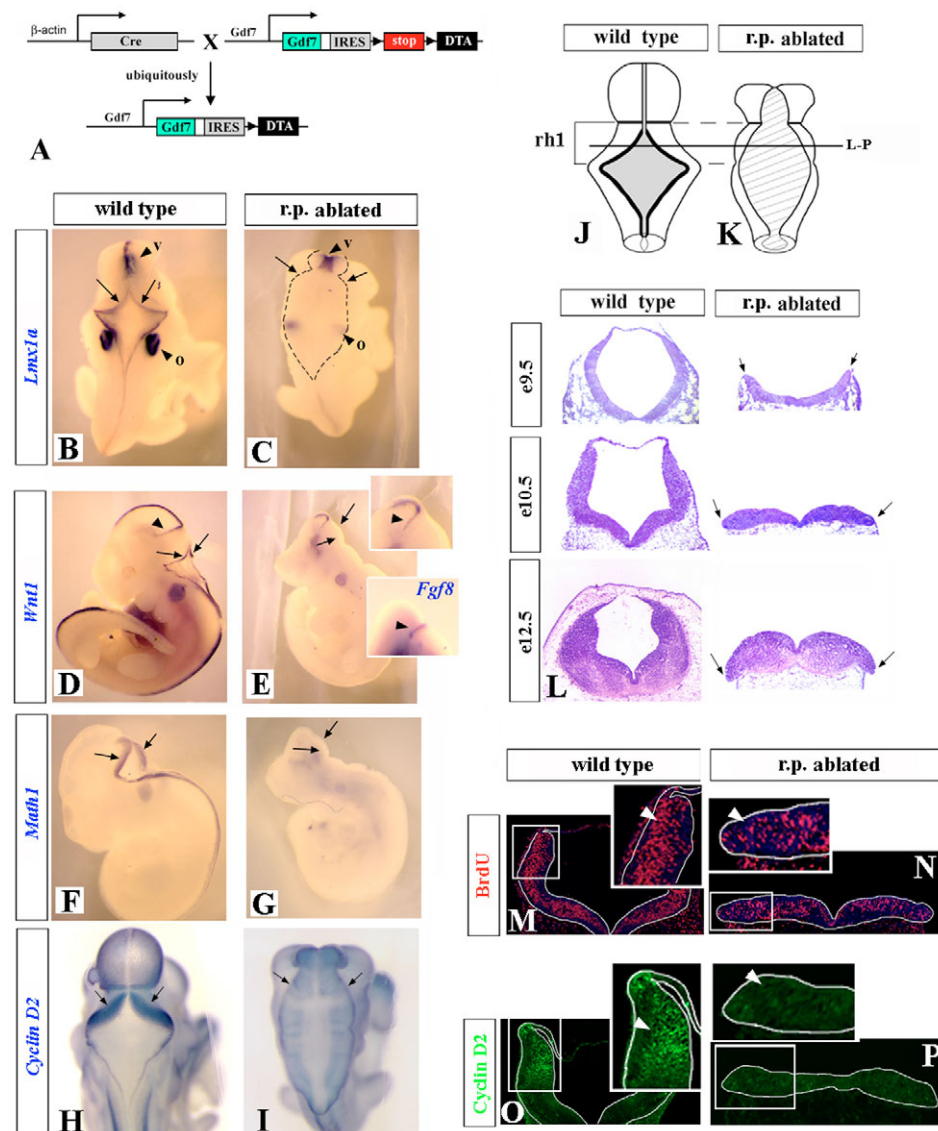
spinal cord, *Wnt1* is sufficient to increase cellular proliferation by activating transcription of members of the cyclin D family (Megason and McMahon, 2002). We noted that cyclin D2 is normally highly expressed in the early cerebellar anlage (Fig. 7H,O). Roof plate ablation was associated with loss of cyclin D2 expression in this region (Fig. 7I,P), suggesting that r1 roof plate at least partially activates cerebellar proliferation by regulating cyclin D2 expression.

## DISCUSSION

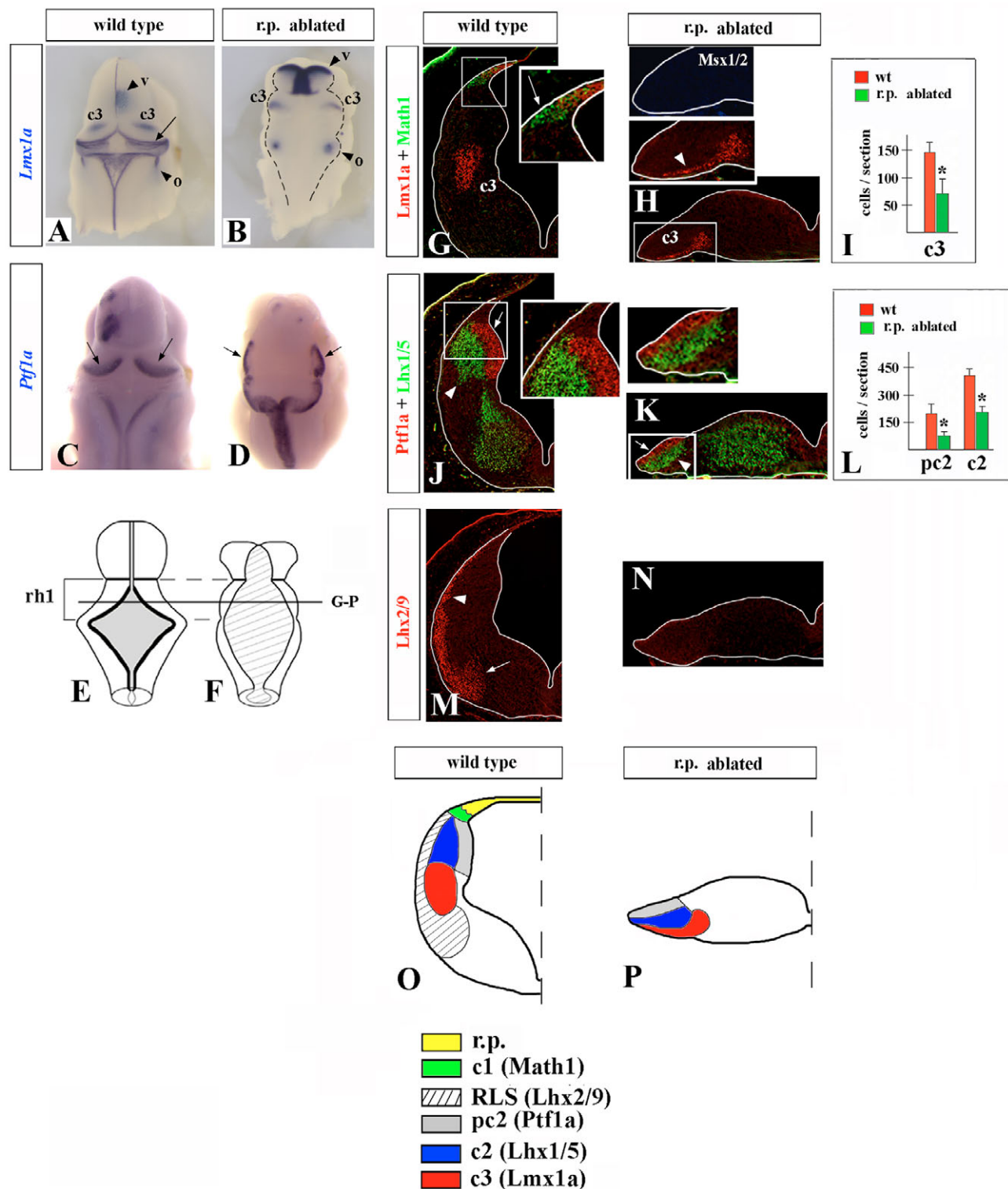
Previous studies have demonstrated that cerebellar development depends on activity of the IsO. In this study we have delineated a gene expression map of the early developing cerebellar anlage and used this as a tool to examine cerebellar anlage patterning mechanisms. Our data establish that the roof plate of r1 acts as an additional signaling center influencing multiple aspects of cerebellar development.

## The r1 roof plate and mechanisms of its formation

There are considerable discrepancies in the literature regarding the definition of roof plate in r1. Here we have defined the roof plate, based on gene expression and lineage analysis, as the *Lmx1a<sup>+</sup>/Gdf7<sup>+</sup>*



**Fig. 7. Roof plate ablation causes loss of *Wnt1* and c1 populations, and reduces cerebellar anlage proliferation at E10.5.** (A) Schematic of the genetic strategy to ablate roof plate cells. (B-I) Whole-mount in situ staining of E10.5 embryos with markers and genotypes indicated. (B,C and H,I) Dorsal views with broken line in C demarking the dorsal edge of the open neural tube. (D-G) Side views. Arrows in B-E indicate position of r1 roof plate. Arrowheads 'o' and 'v' mark normal otic and ventral midbrain *Lmx1a* expression in panels B and C. Upper and lower insets in panel E show *Wnt1* and *Fgf8* isthmus expression. Arrows in panels F and G point to the cerebellar rhombic lip. Arrows in panel H show broad expression of *cyclin D2* which is eliminated in roof plate-ablated embryos (I). Weak staining in I is ventral, visible because of the open neural tube. Dark lines on dorsal edges are artifact. (J,K) Schematic illustration of E10.5 wild-type and roof plate-ablated embryos demonstrating the consequences of ablation. (L) Hematoxylin and eosin-stained sections at the level of the horizontal line in panels J and K in time series. Arrows point to the most dorsal neuroepithelium of roof plate-ablated embryo (M-P) Immunostained transverse sections at E10.5 demonstrating that cell proliferation and cyclin D2 expression is reduced across the entire cerebellar anlage in ablated embryos. Arrowheads point to the developing cerebellar anlage.



**Fig. 8. Effect of roof plate ablation on specification of cerebellar cells at E12.5.** (A–D) Whole-mount in situ staining showing c3 and pc2 populations in wild-type and ablated embryos at E12.5. Arrow in A indicates roof plate expression of *Lmx1a* which is eliminated in panel B. Arrowheads 'o' and 'v' mark normal otic and ventral midbrain *Lmx1a* expression in panels A and B. Arrows in C and D demonstrate pc2-specific expression of *Ptf1a* and the reorientation of the open edges of the dorsal neural tube, illustrated in panels E and F. (G,H,J,K,M,N) Transverse immunostained sections at the level of the horizontal line in panels E and F, with markers and genotypes. c1 and RLS markers are missing in ablated embryos, while pc2, c2 and c3 populations are still present, in reduced numbers and altered positions in the anlage. (I,L) Quantification of pc2, c2 and c3 cells found on transverse sections from wild-type and roof plate-ablated embryos. Bars indicate s.e.m. (O,P) Schematic illustrations summarize gene expression changes.

dorsal domain throughout the anterior/posterior extent of r1 at E10.5. In anterior r1, both *Lmx1a* and *Gdf7* are restricted to a narrow medial domain (Fig. 1J). In posterior r1, they are expressed in both the expanded medial single cell layer and in the edges of the adjacent neuroepithelium, where the *Lmx1a*<sup>+</sup>/*Gdf7*<sup>+</sup> domain significantly overlaps with the *Wnt1*<sup>+</sup> territory (Fig. 1B-E,J,K). Despite the neuroepithelial morphology and ongoing proliferation in this edge domain, it does not express *Math1*, a marker of the rhombic lip, and does not contribute significantly to the adjacent cerebellum. Rather, fate mapping data indicate that *Lmx1a*<sup>+</sup>/*Gdf7*<sup>+</sup> cells (this study) (Currie et al., 2005; Landsberg et al., 2005) and early *Wnt1*<sup>+</sup> cells (Zervas et al., 2004) contribute to the choroid plexus.

Our transgenic overexpression assays demonstrate that a Bmp/*Lmx1a* pathway is sufficient to induce roof plate formation in r1. It is important to note, however, that although early (starting from at least E9.5) overexpression of *Lmx1a* in r1 neuroepithelium generates excessive roof plate, during normal development, c3 cells express the same gene (starting around E11.5-12.5) without acquiring roof plate properties. These data indicate that roof plate-inducing activity of *Lmx1a* is precisely temporally regulated during r1 development.

In the developing spinal cord, *Lmx1a* is absolutely necessary for roof plate development (Millonig et al., 2000). Loss of *Lmx1a* in r1, however, resulted in an interesting roof plate developmental defect instead of its complete loss, as predicted from our spinal cord studies (Millonig et al., 2000). In particular, the anterior roof plate was expanded at the expense of the posterior roof plate in *dreher* r1. *Lmx1a* is therefore required to establish the normal relationship between anterior and posterior r1 roof plate. Chick-quail transplants have shown that the r1 anterior and posterior roof plates have distinct origins. The anterior roof plate is derived from a small dorsomedial population of isthmus-derived cells, which migrate caudally, stopping at the boundary between anterior and posterior r1 (Louvi et al., 2003; Alexandre and Wassef, 2003). Other genes known to influence the extent of r1 anterior roof plate include *Wnt1* and *Otx2* (Louvi et al., 2003), both of which disrupt global isthmus patterning when mutant (Liu and Joyner, 2001). Loss of *Lmx1a*, however, does not influence the positioning of the isthmus or expression of general markers of the mid/hindbrain territory such as *Otx2* and *Gbx2*. It is possible that the low *Lmx1a* expression levels in the anterior roof plate are cell-autonomously required to stop excessive caudal migration of this midline population. Alternatively, high *Lmx1a* levels in the posterior roof plate may normally limit the extent of the anterior roof plate. Further experiments are required to distinguish these mechanisms. Regardless, the presence of residual r1 roof plate in *dreher* embryos indicates that other genes can partially compensate for loss of *Lmx1a*, revealing redundancy in roof plate-inducing mechanisms in r1.

### Roof plate signaling controls specification of the adjacent rhombic lip and its derivative cells, and influences proliferation throughout the entire cerebellar anlage

To understand the role of r1 roof plate in cerebellar patterning, we first developed early markers for cerebellar neuronal progenitors and newly differentiated neurons. By analyzing the expression patterns of additional transcription factors, we determined that the E12.5 cerebellar anlage is divided into several discrete populations (c1-c4), providing the first comprehensive molecular map of the entire early cerebellar anlage. To test the role of the r1 roof plate on specification of these cellular types, we performed gain- and loss-of-function experiments in mice. Induction of ectopic roof plate by *Lmx1a*

expression was associated with significant repatterning of the developing cerebellar anlage. This included induction of c1 cells and their derivative RLS cells (including granule cell progenitors of the external granule layer), and loss of c2-c4 cells and their derivative Purkinje cells and other GABAergic neurons. Loss of c2 and c4 cells can be explained by cell-autonomous conversion of their ventricular zone progenitors into ectopic roof plate cells by exogenous *Lmx1a*. In contrast, the appearance of excessive c1 cells adjacent to the ectopic *Lmx1a*-induced roof plate indicates the non-autonomous patterning activity of the ectopic roof plate. Previously it has been shown that Bmps can induce granule cells when added to naïve r1 neural tissue in vitro (Alder et al., 1999). These studies, however, could not conclusively distinguish whether Bmps directly induce c1 cells or they first induce roof plate structures, which, in turn, produce other, Bmp-unrelated signals, to induce c1 cells and their derivative granule cells. Our explant experiments indicate, however, that Bmps are direct mediators of roof plate signaling in rhombomere 1, since roof plate-dependent induction of *Math1*<sup>+</sup> c1 cells and derivative *Lhx2/9*<sup>+</sup> RLS cells is blocked by the Bmp inhibitors noggin and follistatin. These data are in good agreement with the results of a recent genetic study that showed that Bmp receptors 1a and 1b are required for development of cerebellar granule cells (Qin et al., 2006). At the same time however, the Bmp inhibitors used in our explant experiments significantly blocked, yet did not completely prevent, the appearance of c1 and *Lhx2/9*<sup>+</sup> RLS cells. This suggests that other roof plate-derived secreted factors may also be involved in the development of c1 cells. It is also possible that the repatterning of the cerebellar anlage observed in our *Lmx1a* transgenic embryos is associated with activation of expression of not only *Gdf7*, detected in the current study but also other factors. Since *Lmx1a* activates expression of *Wnt1* in the developing spinal cord (Chizhikov and Millen, 2004a), members of the Wnt family represent good candidates for this activity.

The dependence of c1 induction on roof plate signaling was further demonstrated by analysis of *dreher* and roof plate-ablated embryos. In the *dreher* embryos, which display relatively mild roof plate abnormalities, c1 cells were lost but only in the most anterior domain of r1, further emphasizing differences in patterning mechanisms operating in anterior and posterior r1 dorsal domains. In roof plate-ablated embryos, all c1 cells and their RLS derivatives were completely lost in both the anterior and posterior r1, leading to our conclusion that the roof plate is absolutely required for their specification in vivo.

Surprisingly pc2, c2 and c3 cells were still generated in both *dreher* and roof plate-ablated embryos. These cellular fates are therefore specified independently of roof plate signals. Although this mechanism remains unresolved, absence of c2 and c3 cells from the most anterior region of r1 in wild-type embryos suggests that IsO signaling negatively influences their development.

Although c2 cells and c3 cells appeared at the appropriate time in roof plate-ablated embryos, they were generated in significantly reduced numbers and were displaced. Reduction of these cells was associated with loss of cyclin D2 expression and decreased proliferation of the cerebellar anlage, indicating that the roof plate controls the total number of c2, c3, and probably other cerebellar cells, at least partially by regulating proliferation of the early cerebellar anlage by activating cyclin D2 expression. Dorsal *Wnt1* expression may normally provide this signal. The displacement of c3 cells argues that that roof plate is also required for the normal positioning of this population. This phenotype may be a direct result of loss of roof plate signaling, or may be mediated by loss of the c1-derived RLS cells in roof plate-ablated embryos.

We conclude that the r1 roof plate is an important signaling center required for the specification of directly adjacent c1 cells and their derivative RLS cells. r1 roof plate is not required for initial specification of more distant early cerebellar populations, such as the c2 and c3 cells defined in this study. The roof plate however does regulate the numbers of these distant populations, at least partially, by regulating proliferation of cerebellar progenitors, and controls positions of some of these cells within the developing cerebellar anlage. This contrasts with the situation in the developing spinal cord, where the roof plate is absolutely required for development of the immediately adjacent (dI1) population and two more distant populations (dI2 and dI3) of dorsal interneurons (Lee et al., 2000). Taken together with significant gene expression differences between anterior and posterior r1, our data argue that r1 dorsal cell fates rely on more complex mechanisms than those in posterior CNS axial levels. Our comprehensive gene expression map will be useful for deciphering these mechanisms.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/15/2793/DC1>

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