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The transcription factor Lmx1b maintains *Wnt1* expression within the isthmic organizer

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SUMMARY

Cells in the caudal mesencephalon and rostral metencephalon become organized by signals emanating from the isthmus organizer (IsO). The IsO is associated with the isthmus, a morphological constriction of the neural tube which eventually defines the mesencephalic/metencephalic boundary (MMB). Here we report that the transcription factor *Lmx1b* is expressed and functions in a distinct region of the IsO. *Lmx1b* expression is maintained by the glycoprotein *Fgf8*, a signal capable of mediating IsO signaling. Lmx1b, in turn, maintains the expression of the secreted factor Wnt1. Our conclusions are substantiated by the following: (i) *Lmx1b* mRNA becomes localized to the isthmus immediately after *Fgf8* initiation, (ii) *Wnt1* expression is localized to the *Lmx1b* expression domain, but with slightly later kinetics, (iii) *Fgf8*-soaked beads generate similar domains of expression for *Lmx1b* and *Wnt1* and (iv) retroviral-mediated expression of *Lmx1b* (*Lmx1b/RCAS*) maintains *Wnt1* expression in the mesencephalon. Ectopic *Lmx1b* is insufficient to alter the expression of a number of other genes expressed at the IsO, suggesting that it does not generate a new signaling center. Instead, if we allow *Lmx1b/RCAS*-infected brains to develop longer, we detect changes in mesencephalic morphology. Since both ectopic and endogenous *Lmx1b* expression occurs in regions of the isthmus undergoing morphological changes, it could normally play a role in this process. Furthermore, a similar phenotype is not observed in *Wnt1/RCAS*-infected brains, demonstrating that ectopic *Wnt1* is insufficient to mediate the effect of ectopic *Lmx1b* in our assay. Since Wnt1 function has been linked to the proper segregation of mesencephalic and metencephalic cells, we suggest that Lmx1b and Wnt1 normally function in concert to affect IsO morphogenesis.

Key words: Lmx1b, Wnt1, Fgf8, Isthmic organizer (IsO), Mesencephalon, Metencephalon

INTRODUCTION

Complexity within the central nervous system (CNS) is generated in a stepwise manner. Early inductive signals subdivide the neural plate along the anteroposterior axis and later developmental cascades function within smaller regions to specify a finer degree of pattern (Lumsden and Krumlauf, 1996). The formation of the mesencephalon (the embryological precursor to adult midbrain structures including the tectum) and metencephalon (the embryological precursor to adult anterior hindbrain structures including the cerebellum) is one of the best studied examples of sequential pattern formation (Wassef and Joyner, 1997). Here, inductive events first regionalize the CNS into mesencephalic and metencephalic domains and, then, a signaling center forms near this junction which organizes both regions.

The initial regionalization phase begins during gastrulation and is characterized by the broad induction of several genes in the anterior neural plate (Beddington and Robertson, 1998; Ang and Rossant, 1993). One aspect of regionalization can be delineated by the expression of two transcription factors, *Otx2* and *Gbx2* (Simeone et al., 1992; Millet et al., 1996; Wassarman et al., 1997; Shamim and Mason, 1998; Niss and Leutz, 1998). While both of these genes are expressed in larger domains, *Otx2* and *Gbx2* can be used to define the mesencephalon and metencephalon, respectively (Millet et al., 1996; Hidalgo-Sanchez et al., 1999b; Millet et al., 1999; Broccoli et al., 1999). Their expression domains meet and are mutually exclusive at the future mesencephalic/metencephalic boundary (MMB) (Hidalgo-Sanchez et al., 1999a).

The second phase of pattern formation begins when an organizer is established just caudal to the *Otx2/Gbx2* junction. The isthmic organizer (IsO), so named because it develops in conjunction with a morphological constriction known as the isthmus, is responsible for patterning much of the mesencephalon and metencephalon along the rostrocaudal axis (Puelles et al., 1996; Joyner, 1996; Wassef and Joyner, 1997). When an additional IsO is transplanted rostrally in embryos, rostral mesencephalic and caudal diencephalic cells can be induced to form caudal mesencephalic structures (Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Marin and Puelles, 1994). Similarly, an

additional isthmus transplanted in the caudal metencephalon can direct adjacent cells to form cerebellar structures usually found in more rostral positions (Martinez et al., 1995).

The molecular basis of IsO patterning is only partially characterized. Genetic studies have demonstrated that *Wnt1*, *En1* and *Pax2* are necessary for the proper development of the IsO (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992; Wurst et al., 1994; Brand et al., 1996; Favor et al., 1996; Torres et al., 1996). *Wnt1*, *Pax2* and *En1* transcripts are all initiated in large domains within the mesencephalic/metencephalic region (MMR) during the first phase of development. However, coincident with the formation of the IsO, these genes are refined to areas within and/or adjacent to the isthmus (Bally-Cuif and Wassef, 1994; McMahon et al., 1992; Nornes et al., 1990; Gardner and Barald, 1992).

Fgf8 is a key mediator of this change in expression pattern. *Fgf8* transcription initiates in rostral *Gbx2*-expressing cells at the time that organizing activity first becomes apparent, and then refines to a tight ring of expression centered within the isthmus (Heikinheimo et al., 1994; Crossley and Martin, 1995; Hidalgo-Sanchez et al., 1999a,b; Shamim et al., 1999). Ectopic application of FGF8 in the rostral mesencephalon and caudal diencephalon organize pattern in a manner strikingly similar to rostral isthmic grafts and can generate ectopic expression of *Wnt1*, *En1*, *En2*, *Pax2*, *Pax5* and *Fgf8* itself (Crossley et al., 1996; Funahashi et al., 1999; Shamim et al., 1999). Thus *Fgf8* expression is sufficient to maintain these genes around the isthmus. *Fgf8* is also required for IsO activity, with IsO-dependent structures failing to form in *Fgf8* mutants (Reifers et al., 1998; Meyers et al., 1998). The relationship between Fgf8 and the transcription factors it regulates is only partially understood.

An important aspect of Fgf8 function is its regulation of *Wnt1*, a second secreted factor necessary for IsO activity (McMahon et al., 1992). Once the isthmus forms, *Wnt1* mRNA is maintained in a ring rostral to and partially overlapping *Fgf8*-expressing cells (Bally-Cuif et al., 1995a; Hidalgo-Sanchez et al., 1999a; Shamim et al., 1999). While this expression is known to be Fgf8-dependent (Reifers et al., 1998), the transcription factor(s) mediating the maintenance is unknown.

Wnt1 is also required for Fgf8 maintenance (Lee et al., 1997). While *Fgf8* initiates in $Wnt^{-/-}$ mice, it is not maintained. Whether Wnt1 is sufficient to stimulate *Fgf8* expression has not been tested.

Chick Lmx1b (previously referred to as lmx-1) is a LIM homeodomain protein that is expressed in the CNS, the developing limb buds and the mesonephros. While its role in dorsal pattern formation in the limb is clearly established (Riddle et al., 1995; Vogel et al., 1995a; Chen et al., 1998), less is known about its role in the CNS. Previously, *Lmx1b* was reported to be expressed in developing spinal cord, with its expression resolving into the floor plate, roof plate and a subset of interneurons (Tsuchida et al., 1994; Riddle et al., 1995). However, its role in the formation of more anterior neural structures has not been described.

Here we report that *Lmx1b* has a dynamic expression pattern in the rostral CNS, with expression persisting in large portions of the dorsal and ventral midline as well as the IsO. Within the IsO, our experiments suggest that Lmx1b acts as an effector of Fgf8 in the regulation of *Wnt1*. Furthermore, we test the ability of Lmx1b and Wnt1 to alter the expression of other IsO genes. Our results reveal that Lmx1b regulates the expression of *Wnt1* and suggest that both genes play a role in the morphogenesis of the MMB.

MATERIALS AND METHODS

Chick surgeries and recombinant retroviruses

All experimental manipulations were performed using White Leghorn chick embryos provided by B&E Eggs (Stevens, PA). Chicks were staged according to the Hamburger and Hamilton system (Hamburger and Hamilton, 1951). The *Lmx1b*/RCAS(A) virus has been previously described (Riddle et al., 1995). The *Wnt1*/RCAS(B) virus was constructed by cloning the mouse *Wnt1* cDNA into the RCAS vector. The virus was cultured and concentrated as previously described (Morgan et al., 1992).

In situ hybridization

Embryos were harvested in sterile PBS at the stages indicated and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were processed for whole-mount in situs as previously described (Riddle et al., 1993), and for section in situs as previously described (Shepherd et al., 1996). For section in situs, embryos were sectioned at 12 μM on a cryostat.

Single and double detections were done as described previously using digoxigenin-and fluorescein-labeled cRNA (Riddle et al., 1995). In the case of double detections, both probes were added simultaneously. Next, an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was added. Detection was performed using BCIP/NBT (Molecular Probes) as substrate. For the second detection, alkaline phosphatase activity was inactivated (65°C for 30 minutes), and then an alkaline-phosphatase-conjugated anti-fluorescein antibody was added. After washing, the second probe was detected using BCIP/INT (Molecular Probes) as the color substrate. Thus, the first probe labeled violet, and the second probe labeled orange.

Lmx1b, *Fgf8*, *Gbx2*, *Wnt1* and RCAS probes have been previously described (Crossley and Martin, 1995; Hollyday et al., 1995; Riddle et al., 1995; Niss and Leutz, 1998). An *En1* probe containing the entire coding region was isolated by screening a chick limb bud cDNA library using a genomic fragment. The chick *Pax2* probe was generated by PCR from a stage 22 whole chick embryo library, using the following primers: [5′-ccggatcgatcactgcaaagcagacccctctc-3′ and 5′-ccgggaattccgatcca(a/g)agc(c/t)tcc agctgc-3′] and cycling conditions were: [93°C × 5 minutes{(93°C × 30 seconds)(60°C × 45 seconds)(72°C × 45 seconds) × 30 cycles}72°C × 7 minutes]. The resulting 790 bp fragment was blunt cloned into the *Eco*RV site in pBluescript SK+. The plasmid was sequenced. These data, along with an expression analysis, confirmed the cDNA to be chick *Pax2*.

Antibody staining

Expression of the RCAS virus was detected in sectioned embryos and in whole mount as previously described (Riddle et al., 1995).

Histological analysis

Embryos were harvested in PBS at the times indicated, and the brains were dissected free. Brains were then fixed in 4% paraformaldehyde overnight at 4°C, dehydrated in an ethanol series (30%, 50%, 70%, 80%, 95%, 100%), transferred to xylene, embedded in paraffin and sectioned at 10 μM. Sections were dewaxed, rehydrated and stained with cresyl violet.

Bead implants

Heparin-coated acrylic beads (Sigma) were washed twice in PBS, then split using forceps. Appropriate-sized bead halves were soaked in 0.4

μg/μl FGF8 (R&D Systems) and inserted into the CNS using a sharpened tungsten needle.

RESULTS

Lmx1b expression in the developing brain

As a first step in understanding Lmx1b function in the rostral CNS, we examined its expression. *Lmx1b* expression initiates in the anterior neural plate by stage 6 (Fig. 1A), with highest levels occurring lateral to the midline (Fig. 1A,B). At stage 8, it is highly expressed in the rostral neural folds as they begin to close. By stage 9, *Lmx1b* expression is widespread throughout the caudal forebrain, midbrain and hindbrain (Fig. 1C). At this stage, the highest levels are detected dorsally (red arrow in Fig. 1D).

After this broad initiation, *Lmx1b* expression is maintained in three major areas of the brain. Two of these domains, the dorsal and ventral midline, extend over much of the CNS. Caudally, *Lmx1b* expression eventually extends to the tip of the neural tube, but rostrally it is excluded from a portion of the forebrain. At stage 10, this exclusion is apparent with only the caudal half of the forebrain being *Lmx1b* positive (Fig. 1E). By stage 15, these expression domains have narrowed and are associated with the morphologically distinct regions at the midline (Fig. 1F). By stage 20, these expression domains are contiguous over much of the rostrocaudal axis of the embryo with expression absent from the rostral telencephalon, the rostral diencephalon and middle diencephalon (asterisks in Fig. 1G). This neural expression domain is contiguous with the more caudal floor and roof plate expression patterns previously reported (Riddle et al., 1995).

Beginning at stage 10, and continuing through stage 20, *Lmx1b* is also maintained in a ring centered just rostral to the most constricted portion of the isthmus (see red and black arrowheads, respectively, in Fig. 1E). At stage 10, this ring is broad. During subsequent stages, the rostral limit of this expression domain is not maintained. By stage 15, this ring of *Lmx1b* expression is narrower, with the highest levels centered just rostral to the isthmic constriction (compare red and black arrowheads, respectively in Fig. 1H). After stage 10, *Lmx1b* expression is also detected in the mesenchyme overlying the rostral isthmus (black arrows in Fig. 1I). By stage 20, *Lmx1b* is largely undetected in the neural tissue at the IsO, but persists in the mesenchyme overlying this region (data not shown).

This refinement of *Lmx1b* expression occurs when the isthmus is undergoing a dramatic morphological movement. Classically, the isthmus was thought to delineate the MMB. However, grafting experiments have demonstrated that the caudal border of *Otx2* expression is a better marker for the future MMB (Millet et al., 1996). Therefore, at stage 10, the isthmus lies notably caudal to the MMB. From stages 11-15, morphological movements within the neural tube result in the isthmic constriction moving rostrally, eventually residing slightly caudal to *Otx2* expression (Millet et al., 1996, Hidalgo-

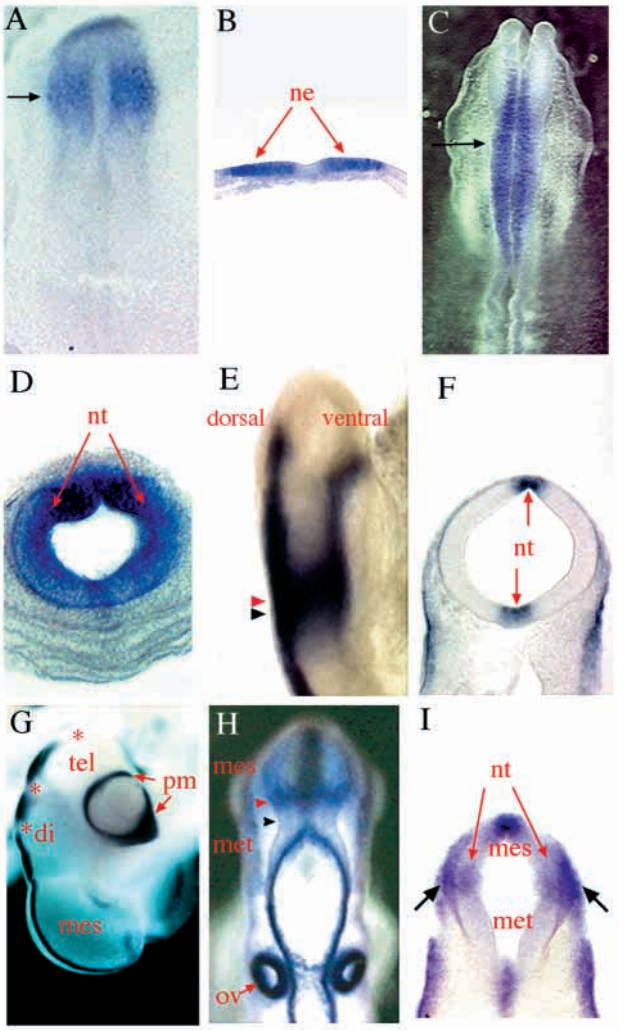


Fig. 1. *Lmx1b* expression in the developing chick brain. In whole-mount panels (A,C,E,G,H), embryos are oriented such that rostral is up; in vibratome-sectioned panels (B,D,F,I), dorsal is up. (A) Dorsal view of stage 6 embryo. *Lmx1b* initiates by stage 6 in the neural plate. An arrow marks the level at which the section for B was taken. (B) At stage 6, *Lmx1b* transcripts are limited to the neuroepithelium and are reduced at the midline. (C) Dorsal view of stage 9 embryo. *Lmx1b* is detected through most of the future brain, excluding the most rostral portions. An arrow marks the level of the position of the section used in D. (D) At this same stage, *Lmx1b* expression is found throughout the neural tube. Expression is robust in the dorsalmost portion of the CNS. (E) Lateral view stage 10 embryo. *Lmx1b* expression persists in the isthmus, dorsal, and ventral midlines. Black arrow head indicates the center of the isthmus. Red arrowhead marks the center of *Lmx1b* expression at the isthmus. (F) Transverse section of stage 15 rostral mesencephalon. *Lmx1b* expression is found in the dorsal and ventral midlines. (G) Lateral view of stage 20 head. *Lmx1b* continues to be expressed throughout the ventral midline, but the dorsal midline expression pattern develops gaps in the mid-diencephalon and at the diencephalon/telencephalon border. Expression also remains absent from the rostral telencephalon (see red asterisks). Periocular mesenchyme (pm) also expresses *Lmx1b.* (H) Dorsal view of stage 15 embryo. *Lmx1b* is expressed in a band just rostral (red arrowhead) to the constricted portion of the isthmus (black arrowhead). Expression also occurs in the otic vesicles (ov). (I) Longitudinal section of a stage 15 embryo through the areas surrounding the isthmus. *Lmx1b* transcripts are also seen in the mesenchyme directly adjacent to the ring of expression in the CNS (black arrows). Expression is also present in the dorsal and ventral midlines of the CNS and more caudal surface epithelium. di, diencephalon; mes, mesencephalon; met, metencephalon; ne, neuroepithelium; nt, neural tube; tel, telencephalon.

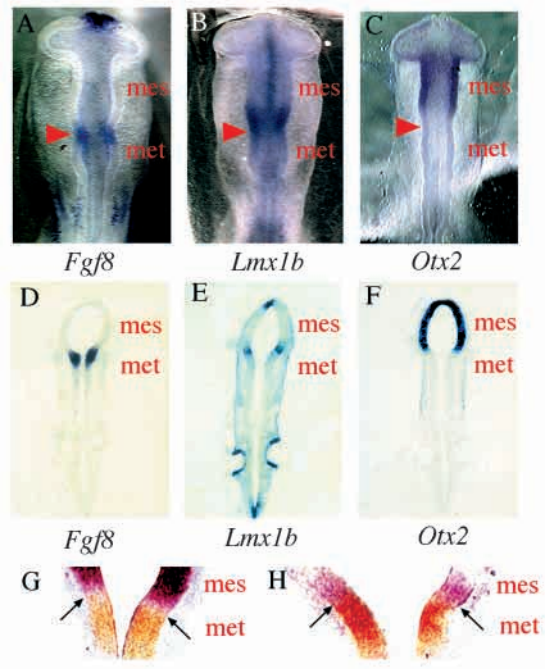


Fig. 2. *Lmx1b* expression overlaps that of *Fgf8* and *Otx2* at the IsO. (A-C) A comparison of *Fgf8*, *Lmx1b*, and *Otx2* expression patterns in stage 10 embryos. Red arrowheads mark the isthmic constriction. (A) *Fgf8* expression is centered at the isthmic constriction. (B) *Lmx1b* expression is also expressed at the constriction, but extends more rostrally. (C) The caudal limit of *Otx2* expression overlaps with *Lmx1b* and abuts *Fgf8*. (D-H) By stage 15, the expression of *Fgf8*, *Lmx1b*, and *Otx2* have refined but *Lmx1b* is still centered in a gap that has developed between *Fgf8* and *Otx2* expression domains. (D-F) Serial sections of a stage 15 embryo showing (D) *Fgf8*, (E) *Lmx1b* and (F) *Otx2* expression domains. (G) Two color section in situ hybridization shows that, by stage 15, a small gap has developed between *Otx2*-expressing cells (violet) and *Fgf8*-expressing cells (orange-yellow). (H) At the same stage, *Lmx1b* expression (violet) is present both within this gap, and more rostral. It also extends caudally into the rostral *Fgf8* expression domain (orange-yellow). In both G and H, an arrow marks the anterior limit of *Fgf8* expression. mes, mesencephalon; met, metencephalon.

Sanchez et al., 1999a). Since *Lmx1b* is expressed precisely within the region of the neural tube undergoing this change, we wanted to compare its expression with *Otx2* and *Fgf8*; markers for the MMB and isthmic constriction, respectively.

Lmx1b expression partially overlaps that of *Fgf8* and *Otx2* from stages 10-20 in the area surrounding the MMB. At stage 10, *Fgf8* and *Otx2* expression domains abut, and possibly overlap (Hidalgo-Sanchez et al., 1999). At this same stage, *Lmx1b* transcripts extend from the caudal *Fgf8* expression region posteriorly into the caudal *Otx2* region anteriorly (Fig. 2A-C). At stage 15, adjacent sections show that *Lmx1b* continues to be expressed near the junction of *Otx2* and *Fgf8* regions (Fig. 2D-F). Using two-color in situ hybridization, a closer examination of this relationship reveals that a small gap has developed between stage 15 *Otx2* and *Fgf8* expression domains in the lateral portions of the neural tube (Fig. 2G). This relationship has been observed by Hidalgo-Sanchez et al. (1999) at stage 20. *Lmx1b* overlaps with the rostral domain of *Fgf8*, and continues into the *Otx2* negative and -positive regions (Fig. 2H). This relationship between *Fgf8*, *Otx2* and *Lmx1b* domains remains qualitatively unchanged until *Lmx1b* expression fades at stage 20 (data not shown). Therefore, *Lmx1b* expression at the isthmus is both (i) temporally and spatially coincident with the morphological change that defines the MMB and (ii) consistent with it being maintained by FGF8 in this region.

Wnt1 expression temporally and spatially follows that of *Lmx1b* at the IsO

Lmx1b and *Wnt1* expression domains overlap in many regions of the CNS. *Lmx1b* expression initiates broadly in the anterior neural plate by stage 6 (Fig. 1A). *Wnt1* expression initiates at stage 7 in a similar, but more restricted, lateral region that will form the dorsal midline after neural tube closure (Hollyday et al., 1995; Shamim et al, 1999). Between stages 9 and 10, *Wnt1* expression spreads to the more ventral regions of the mesencephalon (Shamim et al., 1999), becoming coincident with *Lmx1b* expression in these same regions. At stage 10, *Wnt1* is still expressed in a broad domain while *Lmx1b* has begun to fade in the rostral mesencephalon (Fig. 3A,B). Rostral *Wnt1* expression is unstable and, by stage 12, the expression domain of both genes is similar (compare Fig. 3C with D). From stages

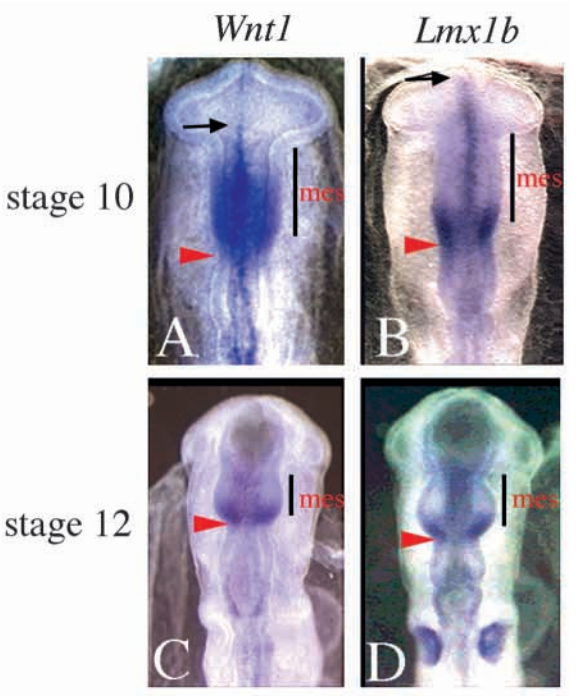


Fig. 3. *Wnt1* expression is maintained in the *Lmx1b* domain after isthmus formation. (A) At stage 10, *Wnt1* expression is still widespread throughout the mesencephalon. A black arrow marks the rostral limit of *Wnt1* expression in the dorsal midline. (B) At the same stage, *Lmx1b* expression has begun to localize to the rostral isthmus. A black arrow marks its rostral limit in the dorsal midline. (C) By stage 12, *Wnt1* expression has become restricted to a ring within the rostral isthmus. (D) At stage 12, *Lmx1b* expression occurs in an identical domain. *Lmx1b* and *Wnt1* expression domains remain localized to the rostral isthmus between stages 12-20 (also see Fig. 5B, F; and data not shown). In all panels, rostral is up. Red arrowheads mark the isthmic constriction in all panels. Black bars mark the approximate extent of the mesencephalic region, which is slightly rostral to the isthmus at these stages. See Fig. 2C; mes, mesencephalon.

12-20, *Wnt1* is maintained in a pattern identical to that of *Lmx1b* at the isthmus, as well as much of the dorsal midline throughout the CNS (data not shown). However, *Lmx1b* dorsal midline expression extends into the telencephalon, while *Wnt1* does not. Nevertheless, using double-label in situ hybridization, we do not observe stable *Wnt1* expression in the absence of concomitant *Lmx1b* expression (data not shown). Thus, the *Lmx1b* expression pattern during stages 10-20 suggests that it could maintain *Wnt1* expression in the rostral isthmus, as well as in the dorsal midline.

Lmx1b and *Wnt1* are similarly responsive to *Fgf8*

The expression pattern of *Fgf8* and its ability to organize the rostrocaudal axis of the mesencephalon suggests that it might regulate *Lmx1b.* To test this, we implanted FGF8-soaked beads into the rostral midbrain and caudal diencephalon at stages 10-11. We observed FGF8-dependent ectopic *Lmx1b* expression in neural tissue as early as three hours after implantation (*n*=10/13) (compare Fig. 4A and B). This expression was observed throughout the neural tube on the implanted side (Fig. 4C). Since *Lmx1b* expression is normally greatly reduced, or absent, in this region of the mesencephalon, FGF8 increased *Lmx1b* mRNA levels and possibly re-initiated *Lmx1b* expression in these cells. Ectopic *Wnt1* was observed in a similar pattern of expression after FGF8 bead implantation (n=8/11) (Fig. 4D). Neither *Wnt1* nor *Lmx1b* was detected beyond the rostral half of the prosencephalon. However, *Lmx1b* expression extended more rostrally (compare Fig. 4B and D).

When we harvested experimental embryos 40-49 hours postimplantation, we found ectopic *Lmx1b* expression near FGF8-soaked beads (compare Fig. 5A with Fig. 5B, *n*=31/32). Areas of ectopic *Lmx1b* were frequently continuous with endogenous *Lmx1b* expression at the dorsal midline. Often *Lmx1b* transcripts formed a ring adjacent to the bead, resembling its normal expression pattern at the isthmus (compare black and red arrowheads, respectively, in Fig. 5B). Sections through induced regions identified *Lmx1b* expression in both the neural tube and overlying mesenchyme (Fig. 5C). By varying the location of implanted FGF8 beads in the anterioposterior axis, we have determined that *Lmx1b* is only induced rostral to the isthmus. This is in contrast to *En1* expression, which can be maintained by FGF8 in the metencephalon (*n*=4/6, Fig. 5E).

Implanting FGF8 beads in the rostral mesencephalon and prosomeres 1 and 2 can yield variability in tectal morphologies (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). In 5% of our embryos, we observe *Lmx1b* expression in a broader pattern across the optic tectum. In these cases, the optic tectum is dramatically smaller on the operated side (Fig. 5D). Regardless of the shape and size of the tectum, ectopic *Lmx1b* transcripts correlate with regions of the midbrain that were morphologically distinct.

The pattern of *Lmx1b* induction in the neural tube is always very similar to ectopic *Wnt1* (compare black arrowheads in Fig. 5B with F). When we probe for the expression of both genes in a single embryo, we never observe *Lmx1b*-independent activation of *Wnt1* in the CNS (data not shown).

Lmx1b can maintain *Wnt1* expression in the absence of *Fgf8*

Since endogenous and ectopic *Wnt1* expression occurs within *Lmx1b* domains in the brain, Lmx1b could regulate *Wnt1* expression. To test this directly, we misexpressed *Lmx1b* in the developing CNS using a replication-competent retroviral vector (*Lmx1b*/RCAS). We injected virus into stage 8-11 neural tubes, harvested the embryos 40-49 hours later, and examined *Wnt1* expression. Lmx1b induced ectopic *Wnt1* expression in the tectum (i.e., dorsal mesencephalon) with 100% penetrance (*n*=30/30, Fig. 6B, compare with A). When these embryos are probed for the location of the virus, we find precise overlap of viral staining and ectopic Wnt1 expression (data not shown). However, in those same embryos, we detected viral staining in other regions of the CNS without accompanying *Wnt1* expression. We additionally assayed for ectopic *Fgf8*, *En1*, *En2*, *Gbx2*, *Otx2* and *Pax2* in infected embryos. At a variety of stages, no change in expression was detected (*n*=4-6 in each case, data not shown). No ectopic expression of these genes or *Wnt1* or *Lmx1b* was seen in control embryos injected with alkaline phosphatase/RCAS (AP/RCAS) (*n*=4-8 in each case, data not shown).

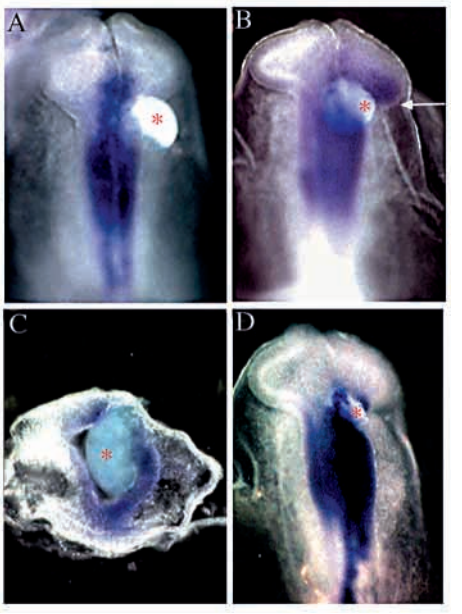


Fig. 4. FGF8 rapidly stimulates ectopic *Lmx1b* and *Wnt1* expression. (A) PBS-soaked beads implanted into prosomere 2 fail to induce *Lmx1b* expression. (B) FGF8-soaked beads generate ectopic *Lmx1b* expression around the bead. The white arrow marks the approximate plane of the vibratome section visualized in C. (C) Ectopic *Lmx1b* transcripts are limited to the neural tissue at this time. (D) *Wnt1* is ectopically expressed in a similar, but smaller domain around an FGF8-soaked bead. Gene expression was visualized using whole-mount in situ hybridization. Red asterisks mark the location of beads in all panels. All embryos were harvested 3 hours after bead implantation.

Wnt1 is unable to affect the expression of other genes localized at the isthmus

To determine whether Wnt1 is able to regulate *Lmx1b* and other IsO-associated genes, we injected a retroviral vector-expressing *Wnt1* (*Wnt1*/RCAS) into the neural tube of stage 9-12 chicks. We incubated the embryos until stages 19-21 (40-48 hours later) and then assayed for ectopic *Lmx1b* expression using whole-mount in situ hybridization. We never detected

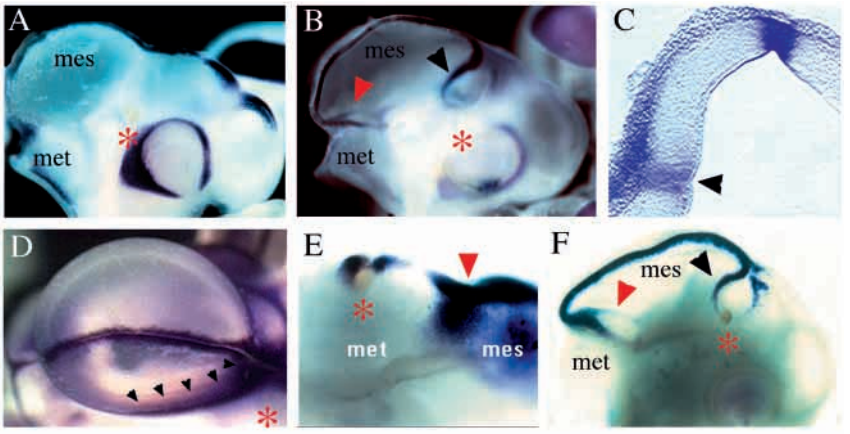


Fig. 5. FGF8-soaked beads induce ectopic *Lmx1b*, *Wnt1* and *En1* in distinct expression domains. In all panels except C, rostral is to the right. A red asterisk marks the location of beads in all panels. (A) Lateral view. Control beads fail to maintain *Lmx1b* expression when implanted into the rostral mesencephalon or caudal diencephalon. (B) Lateral view. FGF8-soaked beads stimulate ectopic *Lmx1b* expression. Often ectopic *Lmx1b* is expressed in a domain (black arrowhead) similar to the endogenous isthmus expression domain (red arrowhead). (C) Transverse section through rostral mesencephalon near an FGF8 bead. Ectopic *Lmx1b* expression (black arrowhead) is found in neural tissue as well as the overlying mesenchyme. Ectopic and endogenous *Lmx1b* expression at the dorsal midline (top of panel) is coincident with a change in neural tube morphology. (D) Dorsal view of stage 20 dorsal mesencephalon (tectum), with left side up, and right side down. Occasionally, a "collapsed tectum" phenotype is generated in which the operated side (bottom half) is much smaller than the contralateral side (compare right and left halves). These smaller tecti correlate with a broader induction of *Lmx1b* (indicated by black arrowheads) near the bead. (E) FGF8-soaked beads implanted posterior to the isthmus (red arrowhead) induce *En1* near the bead. The red arrowhead marks the isthmus. (F) FGF8-soaked beads stimulate ectopic *Wnt1* expression (black arrowhead) in a domain identical to Lmx1b within the mesencephalon, and caudal diencephalon. The red arrowhead marks the endogenous expression of *Wnt1* at the isthmus. Gene expression was visualized using whole-mount in situ hybridization. mes, mesencephalon, met, metencephalon.

ectopic *Lmx1b* anywhere in the brain (*n*=0/6); subsequent probing for the viral mRNA demonstrated that we had generated heavily infected areas in the mesencephalon and metencephalon (data not shown). We also assayed for altered *Fgf8*, *En1*, *En2*, *Pax2*, *Otx2* and *Gbx2* expression (*n*=4-8 infected embryos in each case). We never observed ectopic initiation or maintenance of gene expression in infected regions.

The morphological effects of ectopic Lmx1b and Wnt1 are distinct

To determine whether Lmx1b affects later neural development, we examined the morphology of stage 35 midbrains after *Lmx1b*/RCAS infection. Heavily infected midbrains formed clefts along the ventricular surface of the tectum at the site of infection, with buckling often occurring in adjacent regions (*n*=10/12, compare wild-type embryos in Fig. 7A and D with Fig. 7B,C,E,F). Cresyl violet staining suggests that Lmx1b does not adversely affect the histological organization of the tectum at stage 35 (compare Fig. 7D with F). These clefts often form perpendicular to the midline and can involve a large region. Viewed externally, the ectopic midbrain folds form near infected regions, presumably due to an inward buckling near the clefts (Fig. 7C,E). This effect occurs in the absence of any detected increase in *Fgf8*, *Pax2* or *En1* expression, suggesting that these factors are not required for the phenotype. Control embryos injected with AP/RCAS were indistinguishable from wild type (*n*=0/15).

Given the ability of Lmx1b to maintain *Wnt1*, the tectal phenotype might be an indirect consequence of ectopic *Wnt1* expression. To test this, brains were infected with *Wnt1*/RCAS at stages 8-11 and then assayed for any phenotypic changes similar to *Lmx1b* infected brains at stage 35. Surprisingly, the tecti of all infected animals appear identical to wild type (compare Fig. 8A-C with D-H). Wnt1 is biologically active since the telencephalon is strikingly larger than in wild-type brains. This telencephalic phenotype is apparent on both the alar and basal plates (Fig. 8D,F). We conclude that ectopic Wnt1 is not sufficient to alter midbrain morphology in a manner similar to Lmx1b.

DISCUSSION

Lmx1b is known to function in the formation of a number of embryonic structures. The best studied role for Lmx1b is in limb development, where it specifies dorsal pattern (Riddle et al., 1995; Vogel et al., 1995b; Chen et al., 1998). *Lmx1b* is also necessary for normal kidney development (Chen et al., 1998). Additionally, human *Lmx1b* is mutated in Nail Patella

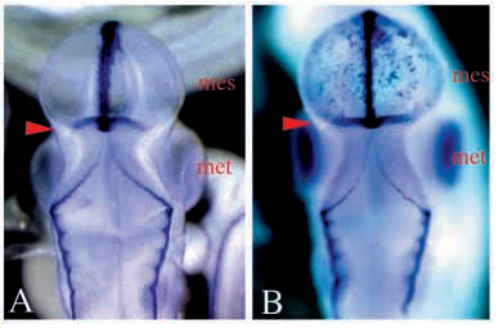


Fig. 6. Ectopic *Lmx1b* can maintain *Wnt1* in the dorsal mesencephalon. Stage 8-11 embryos were injected with *Lmx1b*/RCAS and allowed to develop for 40-49 hours post-infection. (A) Dorsal view of wild-type *Wnt1* expression in stage 21 chick embryo. (B) After *Lmx1b*/RCAS infection, *Wnt1* expression occurs in the dorsal mesencephalon (tectum) lateral to the dorsal midline. The red arrowhead marks the normal ring of *Wnt1* expression. Caudal to the isthmus, no ectopic *Wnt1* is generated. Retroviral infection of metencephalic cells was confirmed by using a cRNA probe to RCAS (data not shown). Gene expression was visualized using whole-mount in situ hybridization. mes, mesencephalon; met, metencephalon.

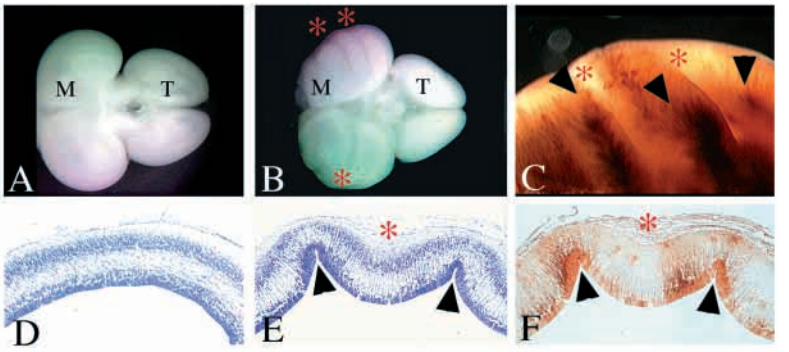


Fig. 7. Ectopic expression of *Lmx1b* alters tectal morphology. *Lmx1b*/RCAS was injected into stage 8-11 neural tubes. (A) Dorsal view of wild-type E8.5 brain. (B) After ectopic *Lmx1b* expression, the tectum (dorsal mesencephalon) often formed inward "folds" which were transverse to the rostrocaudal axis (asterisks). (C) Using a mAb directed against the retroviral gag protein (3c2), broad domains of viral infection (black arrowheads) are often found adjacent to areas of folding (asterisks). (D) Parasagittal sections of a wild type (E8.5). (E) Parasagittal section through an *Lmx1b* infected brain (E8.5). While clefts are present on the ventricular side (arrowheads), all of the tectal layers are present at this stage. (F) Adjacent section of E. After using the antibody 3c2 in immunohistochemistry, clefts (arrowheads) are found to occur in regions of broad retroviral infections. Folds (asterisks) form in areas between these clefts. Sections in D and E were stained with cresyl violet. m, mesencephalon; t, telencephalon.

Syndrome (NPS), a disease associated with abnormal limb, eye and kidney development (Dreyer et al., 1998).

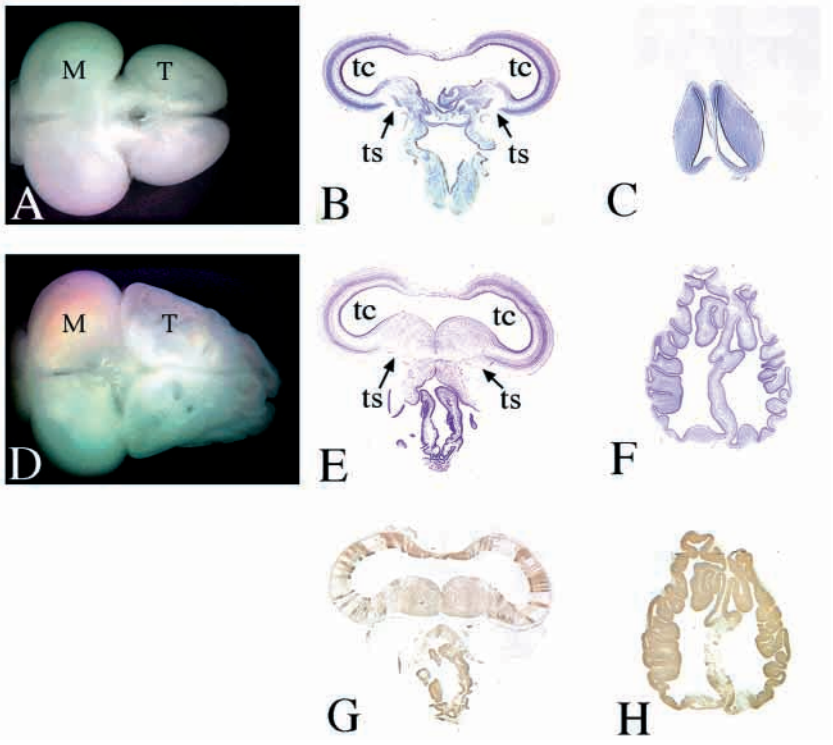
The role of Lmx1b in anterior CNS development has not been reported. Our work reveals that *Lmx1b* is also expressed in the developing brain and addresses its function at the IsO. We find that its function in this region is specifically linked to the regulation of *Wnt1*. These studies provide unique insight into understanding the genetic hierarchy regulating pattern formation in the IsO.

Lmx1b expression and function in the CNS

Lmx1b expression is dynamic in the developing CNS. It initiates by stage 6, through an undefined mechanism. At stage 10, *Lmx1b* expression quickly fades throughout much of the rostral neural tube, but persists in four distinct domains. Two expression domains occur at the dorsal and ventral midlines. *Lmx1b* is also expressed in a subset of interneurons along the rostral caudal axis of the developing spinal column (Riddle et al., 1995). Finally, *Lmx1b* expression persists at the rostral isthmus. Its expression in each of these domains is likely to be regulated by distinct factors.

This research has focused on Lmx1b function at the rostral IsO. Here, high levels of *Lmx1b* expression are observed in a ring of neuroectoderm encompassing the MMB, as defined by the caudal expression of *Otx2* expression. Since *Otx2*-expressing cells adopt mesencephalic fates (Millet et al., 1996, 1999), and only a subset of *Lmx1b*-expressing cells express *Otx2*, *Lmx1b*-positive cells are likely to give rise to both mesencephalic and metencephalic cell types.

This region of *Lmx1b* expression at the IsO has two distinct characteristics. First, it is identical to the *Wnt1* expression domain. As a result, the regulation of *Wnt1* and *Lmx1b* during this phase of development could be linked. Second, this region of the neural tube undergoes a morphological change such that the isthmic constriction appears to tighten and move rostrally (Millet et al., 1996, Puelles et al., 1996). Since *Lmx1b* expression remains centered within the region undergoing this change, it could also play a role in this morphogenesis.



Lmx1b regulation at the MMB

Our data indicate that FGF8 positively regulates *Lmx1b* expression at the MMB. *Lmx1b* expression begins to fade in the rostral mesencephalon by stage 10, but it persists at the isthmus following

Fig. 8. Ectopic *Wnt1* has a dramatic affect on forebrain size, but not tectal morphology. Stage 9-12 embryos were infected with *Wnt1*/RCAS and allowed to develop to E8.5. (A) Dorsal view of wild-type E8.5 brain. (B) Coronal sections through the mesencephalon of wild-type brains. Black arrows mark the torus semicircularis. (C) Coronal sections through the telencephalon of wild-type brains. (D) Dorsal view of *Wnt1*-infected brains (E8.5). Mesencephalic morphology is identical to wild type. However, these brains show grossly enlarged telencephalons when compared to wild type (A). (E) Midbrain coronal sections show that *Wnt1* infected brains have a normal tectum and torus semicircularis (black arrows). (F) Coronal sections of infected forebrains display an enlarge ventricle and disturbed architecture. (G,H) Adjacent sections to E and F, respectively, and they show 3c2 antibody detection against viral coat protein. Sections in B, C, E and F were stained with cresyl violet. m, mesencephalon; t, telencephalon; tc, tectum; ts, torus semicircularis.

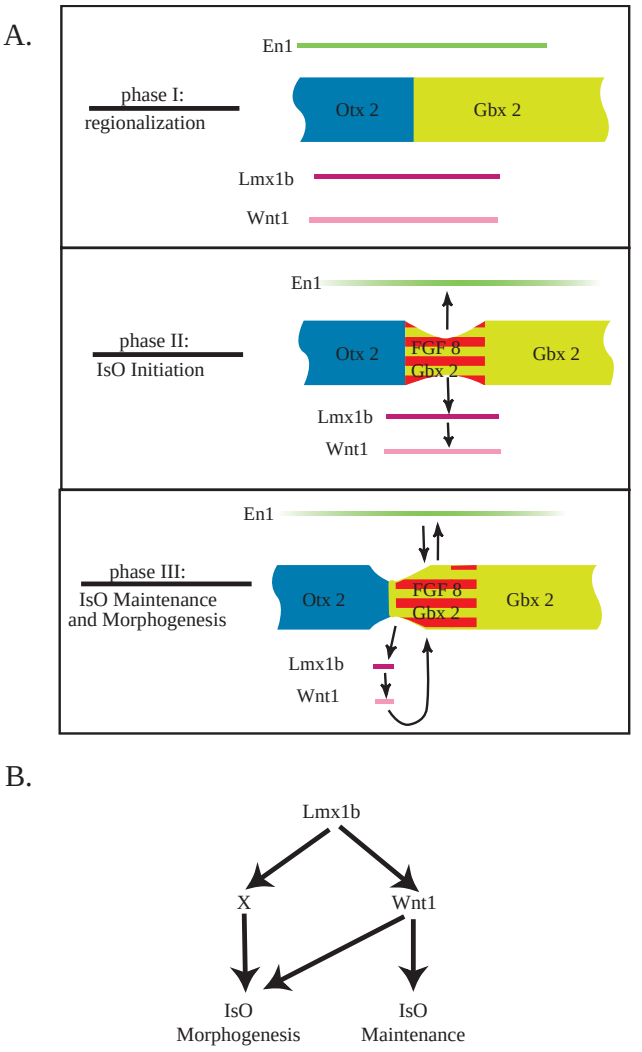


Fig. 9. A model for Lmx1b and Wnt1 function at the chick IsO. (A) Pattern formation at the IsO can be divided into three phases. During phase I (regionalization), *Otx2* and *Gbx2* expression domains form, and identify mesencephalic and metencephalic cells, respectively. The MMB border is defined by the juxtapostion of these two expression domains. A number of genes including *Wnt1*, *En1* and *Lmx1b* are initiated in a broad domain encompassing the MMB. At the beginning of phase II (IsO initiation), Fgf8 expression initiates in the rostral metencephalon (stage 9) and is centered at the nascent isthmus. At this point, the maintenance of *Wnt1*, *Lmx1b* and *En1* become FGF8-dependant. During phase III (IsO maintenance and morphogenesis), *Lmx1b* and *Wnt1* expression refine to a ring in the rostral isthmus. At this point, Lmx1b and Wnt1 maintain *Fgf8* expression. In contrast, *En1* expression persists with highest levels centered at the isthmus, and graded expression observed in the adjacent cells. *En1* also maintains *FGF8*, but potentially regulates it through a distinct mechanism (Shamim et al., 1999). Concomitant with this maintenance (stages 10-15), the isthmic constriction shifts rostrally such that it is coincident with the MMB. During this movement, *Lmx1b* and *Wnt1* expression remains centered within the region of the ishmus undergoing the change. This region is also centered at the small gap that develops between *Otx2* and *Fgf8* expression domains. (B) Lmx1b is sufficient to maintain *Wnt1* expression and alter tectal morphology. We propose that Lmx1b could function both in the maintenance of IsO activity (via its regulation of *Wnt1)* and IsO morphogenesis (via the regulation of *Wnt1* and additional factors). Wnt1 is likely to be a component in both of these functions, but an additional factor(s), x, is required for morphogenesis since ectopic *Wnt1* is unable to phenocopy ectopic *Lmx1b*.

the initiation of *Fgf8* gene expression (stage 9). Additionally, beads soaked in FGF8 maintain *Lmx1b* expression both in the neuroectoderm and head mesenchyme. Neural regulation can be seen within 3 hours, suggesting that the effect may be direct. Furthermore, after 2 days, ectopic neural *Lmx1b* expression refines into a narrow band contiguous with the endogenous roof plate expression. This pattern of expression is very similar to that seen at the isthmus.

Based on the expression data, FGF8 is most likely responsible for the maintenance, rather than initiation of *Lmx1b* in the IsO. Nevertheless, FGF8-soaked beads stimulate ectopic *Lmx1b* transcripts in the stage 10 lateral prosencephalon; an area in which *Lmx1b* is not detected endogenously (Fig. 4B). In this case, FGF8 may be reinitiating *Lmx1b* expression in this region. Alternatively, FGF8 may upregulate *Lmx1b* transcripts from levels present below detection. These results further suggest that FGF8 maintenance of *Lmx1b* expression is an active process at the IsO.

FGF8 is likely to require additional factors to regulate *Lmx1b*, since its ability to do so is restricted along the rostrocaudal axis. FGF8-soaked beads maintain *Lmx1b* rostral to the isthmus and caudal to prosomere three. This could be due to the fact that: (i) FGF8 maintains *Lmx1b* only in the presence of other localized factors, (ii) other factors can abrogate the effect of FGF8 caudal to the isthmus and rostral to prosomere two, or (iii) FGF8 maintains *Lmx1b* only in cells which have previously been made competent to respond. Notably, the area where FGF8 can regulate *Lmx1b* is the same region FGF8 can induce isthmic structures (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999).

Lmx1b as a regulator of *Wnt1* expression

Lmx1b and *Wnt1* have strikingly similar endogenous expression patterns in the anterior CNS. *Lmx1b* initiates in a broad domain in the MMR by stage 6, and *Wnt1* expression is initiated in a subset of these cells at stage 7 (Hollyday et al., 1995). From this stage onward, stable *Wnt1* expression occurs within the *Lmx1b* domain. Our research has focused on their relationship during IsO formation and maintenance. Initially, *Wnt1* expression fades in the rostral mesencephalon at a slightly later stage than *Lmx1b* (stage 10 versus stage 12) but, from stage 12-20, they are expressed in an identical domain within the isthmus.

This expression pattern is consistent with Lmx1b maintaining *Wnt1* expression in this region of the brain. Injection of *Lmx1b*/RCAS into the neural tube of stage 8-11 embryos results in ectopic *Wnt1* transcription in the mesencephalon. Since *Wnt1* expression normally fades in all but the most caudal portions of this region by stage 12, and endogenous *Wnt1* and *Lmx1b* expression is colocalized at the isthmus during stages 12-20, our ectopic expression experiments argue that Lmx1b is sufficient to maintain *Wnt1* within the rostral IsO.

The temporal and spatial relationship between the early expression patterns of these genes also suggests that Lmx1b could regulate *Wnt1* expression at stages prior to IsO

formation. However, due to the time of injection (stage 8-11), our experiments are unable to address this role. Loss-of-function experiments demonstrate that *Wnt1* initiates in *Lmx1b*$^{-/-}$ mice, but fails to be maintained at the IsO by E 9.5 (R. Johnson, personal communication). These results argue that, if Lmx1b does play a role in the earliest stages of *Wnt1* expression, it does so in cooperation with other factors. Nevertheless, these gain- and loss-of-function experiments clearly demonstrate that *Lmx1b* is both necessary and sufficient to maintain *Wnt1* expression within the IsO.

While our data indicate that Lmx1b maintains *Wnt1* expression at the IsO, we do not know whether this regulation is direct or unique. Pax2 has previously been suggested to regulate *Wnt1* expression in zebrafish (Kelly and Moon, 1995). As such, *Pax2* could be downstream of Lmx1b regulation. However, we do not detect ectopic *Pax2* expression after *Lmx1b* injection. As a result, any effect of Pax2 on *Wnt1* must occur either upstream of Lmx1b or through a parallel mechanism.

Wnt1 function in the IsO

Although *Wnt1* is necessary for IsO function (McMahon et al., 1992), its role is unclear. Our gain-of-function experiments suggest that Wnt1 is insufficient to alter gene expression or pattern formation within the MMR. However, Wnt1 could still direct these processes, in cooperation with other factors like FGF8. Finally, Wnt1 alone may be sufficient to direct these processes but only within a spatial and temporal context our experiments fail to detect.

Induction and maintenance of the IsO

IsO formation marks a distinct phase in patterning of the mesencephalon and metencephalon. It is characterized by the formation of the isthmus and the refinement of gene expression in or adjacent to this region. During development, a number of genes expressed at the IsO are maintained and the isthmus undergoes a morphological movement such that it coincides with the MMB. A key to understanding the development of this region of the brain is the identification of the factors that play a role in both of these processes.

Pattern generated by the IsO is dependent on the orchestrated function of a number of genes. *Fgf8*, *Wnt1*, *En1*, *En2*, *Lmx1b*, *Pax2*, *Otx2* and *Gbx2* are all expressed in overlapping temporal and spatial domains within this signaling center (Crossley and Martin, 1995; Wassarman et al., 1997; Niss and Leutz, 1998; Shamim and Mason, 1998; Gardner and Barald, 1992; Nornes et al., 1990; Bally-Cuif et al., 1992, 1995b; Simeone et al., 1992; McMahon and Bradley, 1990; and this paper). Loss-of-function studies of these genes demonstrate that each is required for the subsequent development of the mesencephalon and metencephalon (McMahon and Bradley, 1990; McMahon et al., 1992; Meyers et al., 1998; Reifers et al., 1998; Millen et al., 1994; Wurst et al., 1994; Favor et al., 1996; Torres et al., 1996; Acampora et al., 1995; Ang et al., 1996; Wassarman et al., 1997; Brand et al., 1996; R. Johnson, personal communication). In the absence of each, IsO activity is disrupted.

The mechanism by which IsO function fails in each mutant is unclear. A particular factor could participate in IsO signaling by directing rostrocaudal polarity and cell fate. Alternatively, a factor could play a more specific, yet vital role within a cell type. Examples of this would include regulating mitosis or morphogenesis.

Similar phenotypes produced by homozygous null mutations in each of these genes has made their distinctive roles difficult to assess. However, gain-of-function experiments have generated unique insight into the activity of certain factors by revealing what they are sufficient to accomplish. Together, the role of each gene at the IsO becomes clearer.

FGF8 is sufficient to respecify rostral mesencephalic and prosomeres 1 and 2 to form complex caudal mesencephalic structures, as well as structures arising from the isthmus. Our gain-of-function experiments suggest that Fgf8 maintains *Lmx1b*, which in turn maintains *Wnt1*. However, neither *Lmx1b* nor Wnt1 are sufficient to phenocopy Fgf8-mediated effects, arguing that neither is sufficient to mediate IsO signaling.

Our data also suggest that Lmx1b is insufficient to alter cell fate directly in the rostral IsO. It was unable to alter the expression of a number of other IsO genes, including *En1* or *En2*. This is significant since these two transcription factors have been shown to be sufficient to alter tectal polarity, as assayed by Ephrin A2 and A5 expression (Logan et al., 1996; Shigetani et al., 1997). These glycoproteins are expressed in a caudal-to-rostral gradient and are key effectors in the generation of a functional optic tectum (Cheng et al., 1995). We also failed to detect changes in Ephrin A2 and A5 levels after ectopic Lmx1b expression (data not shown). In the absence of an Lmx1b-mediated effect on any other gene besides *Wnt1*, we suggest a more specialized role for its function.

This result is in contrast to previous studies suggesting that *En1* is a target of Wnt1 signaling. Loss-of-function experiments have established the Wnt1 dependency of *En1*-expressing cells (McMahon et al., 1992). Moreover, *Wnt1* expression is largely unnecessary if *En1* is regulated by the *Wnt1* promoter (Danielian and McMahon, 1996). The simplest interpretation of these results is that Wnt1 maintenance of *En1* is direct. However, we do not observe ectopic *En1* expression after either *Lmx1b* or *Wnt1* expression. This result suggests that Wnt1 is necessary for *En1* expression, but not sufficient. Furthermore, our data demonstrate that FGF8 can induce metencephalic *En1* expression in the absence of ectopic *Wnt1* or *Lmx1b*. As such, Wnt1 regulation of *En1* could require Fgf8 as an intermediate. In combination, these studies suggest that FGF8 regulates *En1* and *Lmx1b/Wnt1* by distinct mechanisms.

Lmx1b and IsO morphogenesis

Our data indicate that Lmx1b may affect isthmus morphogenesis. During stages 10-15, *Lmx1b* expression precedes the rostral movement of the isthmic constriction. By stage 16, this movement results in the coincidence of the isthmic constriction and the MMB (Millet et al., 1996). Furthermore, ectopic expression of *Lmx1b* results in alteration in tectal morphology reminiscent of that occurring within the isthmus.

This role for Lmx1b is substantiated by the finding that Wnt1 plays a role in the morphogenesis of the isthmus. A tight border of *Otx2* expression fails to resolve at the isthmus in mice containing the hypomorphic allele of *Wnt1*, *Swaying* (Bally-Cuif et al., 1995a). Furthermore, while *Wnt1*$^{-/-}$ mice are partially rescued by a transgene directing *En1* expression under the control of the *Wnt1* promoter, these mice fail to form an

isthmus (Danielian and McMahon, 1996). Therefore, Wnt1 is necessary for this aspect of IsO function. However, ectopic *Wnt1* does not change the structure of the mesencephalon, suggesting that it is not sufficient to direct this morphogenesis.

Since Lmx1b maintains *Wnt1*, its effect on morphology could simply be an indirect result of the upregulation of that secreted factor. We think this unlikely since ectopic Wnt1 could not mimic the Lmx1b phenotype. Instead, Lmx1b could affect morphogenesis in the isthmus by regulating both *Wnt1* and other factors which, in total, are sufficient to direct the rostral movement of the isthmus. A model describing Lmx1b function at the IsO is presented in Fig. 9.

Lmx1b may also play a larger role in morphogenesis. *Lmx1b* is expressed in highest levels in many regions of the brain associated with changes in morphology including the isthmus, dorsal and ventral midlines, and the neural folds as the neural plate becomes a tube (data not shown). Future studies are required to test its ability to regulate morphology as well as further test its ability to affect neuronal specificity.

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