Wnt signaling is required at distinct stages of development for the induction of the posterior forebrain

Michelle M. Braun[1], Alton Etheridge[2], Amy Bernard, Christie P. Robertson[1] and Henk Roelink[1,2,*]

[1]Program in Neurobiology and Behavior, Department of Biological Structure, Box 357420, University of Washington, Seattle, WA 98195, USA
[2]Program in MCB, Department of Biological Structure, Box 357420, University of Washington, Seattle, WA 98195, USA
*Author for correspondence (e-mail: roelink@u.washington.edu)

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Summary

One of the earliest manifestations of anteroposterior pattering in the developing brain is the restricted expression of *Six3* and *Irx3* in the anterior and posterior forebrain, respectively. Consistent with the role of Wnts as posteriorizing agents in neural tissue, we found that Wnt signaling was sufficient to induce *Irx3* and repress *Six3* expression in forebrain explants. The position of the zona limitans intrathalamica (zli), a boundary-cell population that develops between the ventral (vT) and dorsal thalamus (dT), is predicted by the apposition of *Six3* and *Irx3* expression domains. The expression patterns of several inductive molecules are limited by the zli, including Wnt3, which is expressed posterior to the zli in the dT. Wnt3 and Wnt3a were sufficient to induce the dT marker *Gbx2* exclusively in explants isolated posterior to the presumptive zli. Blocking the Wnt response allowed the induction of the vT-specific marker *Dlx2* in prospective dT tissue. Misexpression of *Six3* in the dT induced *Dlx2* expression and inhibited the expression of both *Gbx2* and *Wnt3*. These results demonstrate a dual role for Wnt signaling in forebrain development. First, Wnts directed the initial expression of *Irx3* and repression of *Six3* in the forebrain, delineating posterior and anterior forebrain domains. Later, continued Wnt signaling resulted in the induction of dT specific markers, but only in tissues that expressed *Irx3*.

Key words: Chick, Forebrain, Embryonic induction, Zona limitans, intrathalamica, Wnt, Dickkopf 1

Introduction

The vertebrate forebrain develops from the anterior-most part of the neural tube, the prosencephalon. As development proceeds, a series of morphological constrictions give way to nested gene-expression patterns in subregions of the forebrain that have distinct developmental fates. Based on these observations, the forebrain is thought to develop from a series of six subregions, called prosomeres, numbered p1-p6 in a caudal to rostral fashion (Rubenstein et al., 1994). However, despite some similarities with the rhombomeres of the hindbrain, several studies show that prosomeres are not true compartments. Most importantly, individual prosomeres do not have the restricted cell lineages and border cell populations that are characteristic of rhombomere segmentation (Golden and Cepko, 1996; Larsen et al., 2001).

An exception to these findings occurs in the developing forebrain, at the zona limitans intrathalamica (zli), the interface between the future, anteriorly located ventral thalamus (vT) and the posteriorly located dorsal thalamus (dT). Before its overt formation, the location of the prospective zli is demarcated by the adjacent but nonoverlapping expression patterns of *Six3* and *Irx3*, which encode Iroquois-type transcription factors. *Six3* is expressed in neural tissue overlying the prechordal plate and *Irx3* is expressed above the anterior-most portion of the notochord. In HH stage 8 chick embryos, the expression of either *Six3* or *Irx3* can confer anterior or posterior identity, respectively, on the developing forebrain by determining the competency of this neural tissue to differentially respond to Fgf and Shh signals (Kobayashi et al., 2002). A question that arises from these studies is how the expression domains of *Six3* and *Irx3* are established.

Several lines of investigation demonstrate that Wnt signaling in early forebrain tissue induces differentiation of the posterior forebrain (van de Water et al., 2001), whereas the absence of Wnt signaling allows differentiation of the anterior forebrain (Mukhopadhyay et al., 2001; Houart, 2002). These studies indicate a role for Wnts in the early anteroposterior patterning of the brain (Nordstrom et al., 2002). Our data extend these observations by demonstrating that Wnt signaling is sufficient to induce *Irx3* expression and suppress *Six3* expression in explanted forebrain tissue. The source of this Wnt activity remains unclear. However, somewhat later in development, Wnts are expressed in and posterior to the zli.

The zli is the first forebrain subdivision to establish, forming above the transition between the notochord and prechordal plate (Figdor and Stern, 1993). The site of zli formation is characterized by the absence of *lunatic fringe* expression (Zeltser et al., 2001). The zli, a narrow strip of tissue that both expresses boundary cell markers and restricts the mixing of cell lineages (Larsen et al., 2001), defines the border between the future dT and vT. Although the role of the zli is unknown, it serves as either the site or limit of expression of several molecules with inductive capacities. *Wnt3a* expression deviates from its pattern along the dorsal neural tube to form a finger-

like projection that extends ventrally at the zli. *Wnt3*, a Wnt family member with 91% identity to *Wnt3a*, is expressed in the prospective dT, with its anterior limit of expression abutting the zli (Roelink and Nusse, 1991; Salinas and Nusse, 1992). In addition to Wnts, the expression patterns of several transcription factors and cell adhesion molecules have sharp borders at the zli. *Gbx2* is expressed posterior to the zli in the dT (Bulfone et al., 1993) and the zli marks the posterior limit of expression for the vT markers *Dlx2*, R-cadherin and cadherin8 (Larsen et al., 2001; Price et al., 1991; Redies and Takeichi, 1996). Based on the timing of *Wnt3* and *Wnt3a* expression, zli-restricted Wnts cannot account for the initial restriction of *Six3* and *Irx3* expression.

Here, we demonstrate that activation of the canonical Wnt signaling pathway is sufficient and required to induce dT-specific gene expression, and that the absence of Wnt signaling allows vT-specific differentiation. Blocking the Wnt response resulted in vT-specific gene expression in dT explants, and exposure of vT explants to Wnt3 resulted in the induction of both early (*Irx3*) and late (*Gbx2*) dT-specific gene expression. Furthermore, misexpression of either *Six3* or the Wnt inhibitor Dkk1 in the presumptive dT initiated differentiation appropriate for the vT. These results indicate that, by determining the domains of *Irx3* and *Six3* expression, Wnt signaling is crucially important for the initial anteroposterior organization of the forebrain. Our observation that Wnts induced *Irx3*, which, in turn, allowed the dT-specific response, indicates that Wnt signaling is required at multiple stages of development of the posterior forebrain.

Materials and methods

Headfold, zli, and prospective dT and vT explant dissection

Headfold explants containing the anterior neural expression domains of *Six3* and *Irx3* were dissected from chicken embryos at HH stage 7-8 (Hamburger and Hamilton, 1992). Extraneous anterior endoderm was trimmed from the headfold tissue prior to culture. For co-culture and prospective dT/vT explant experiments, neural explants were taken from HH stage 8 and stage 17 chicken embryos using a modified protocol from (Yamada et al., 1993). Following dispase (Sigma) proteolysis, neural tissue was isolated and cultured as described below. The anterior limit of the notochord was used as a reference point for dissecting prospective dT and prospective vT explants. Neural explants dissected rostral to the limit of the notochord correspond to prospective vT explants, whereas neural explants dissected just caudal to the limit of the notochord are termed prospective dT explants (Fig. 3A). Explants of the zli were obtained from HH stage-17 embryos, with the excised tissue corresponding to the *Wnt3/3a*- and Shh-expressing region that lies within and just posterior to the zli (Fig. 5A).

Culture conditions and tissue fixation

Explants were embedded in collagen (Collagen Biomaterials) (Yamada et al., 1993) and cultured for 36-48 hours in neurobasal media (Gibco BRL) supplemented with 1% each of penicillin-streptomycin (Gibco BRL), nonessential amino acids (Gibco BRL), glutamine (Gibco BRL), N3 (Yamada et al., 1993) and dextrose (EM Science).

In headfold experiments, the explants were oriented in a rosette with the anterior end of the explant facing out. This orientation allowed both the anterior and posterior portion of the explants to be scored for the expression of markers following culture.

For co-culture experiments, zli explants and explants of prospective dT and vT were incubated either alone or together in collagen. To distinguish zli tissue from prospective dT or vT explant tissue, CellTracker Blue CMAC dye (Molecular Probes) was used to fluorescently label the prospective dT/vT explants according to the manufacturer's instructions. During fixation, the co-cultured explants were visualized and photographed by both fluorescent and light microscopy. These images were merged in Photoshop (Adobe) to create a map of prospective dT/vT versus zli explant tissue for each co-culture condition, thus allowing us to discriminate between zli and prospective diencephalic explant tissue (data not shown).

Wnt was supplied to headfold explants via a soluble Wnt3a-containing supernatant, generated by growing mouse fibroblast L cells stably transfected with a Wnt3a-expression construct (Shibamoto et al., 1998) in Optimem (Gibco BRL) for 4 days. Control supernatant was obtained from mock-transfected L cells. The supernatants were added to explants at a 1:1 ratio with complete neurobasal media.

In prospective dT/vT explant experiments, Wnt was provided by one of two means, with similar results obtained using either Wnt source. First, prospective dT/vT explants were grown on a monolayer of either RatB1A cells or RatB1A cells expressing Wnt3 (Shimizu et al., 1997) in supplemented neurobasal media (see above). Following culture, the explants were placed in collagen, fixed, and processed by in situ hybridization. Alternatively, explants were exposed to the Wnt3a supernatants described above.

To inhibit Wnt signaling in vitro, casein kinase inhibitor 7 (cki7) (Seikasaku America) dissolved in DMSO was added at a final concentration of 50 μM at the start of culture. A similar dilution of DMSO was added to control wells. Alternatively, a Dkk1 supernatant was used to block Wnt signaling in headfold culture experiments. Dkk1 supernatant was generated by growing mouse 293T cells transfected with *pRK5-Dkk1* in Optimem (Gibco BRL) for 48 hours. Control supernatant was obtained from mock-transfected 293T cells transfected with *pRK5* alone. The supernatants were concentrated 10-fold by filtration through 10KNMWL exclusion membranes (Amicon Ultra-15), and added to explants at a 1:1 ratio with complete neurobasal media.

Explants and embryos to be processed by in situ hybridization were fixed overnight in 4% paraformaldehyde in either PBS or MEM, pH 7.4 at 4°C. After fixation, embryos to be sectioned were rinsed in DEPC PBS, followed by 30% sucrose in DEPC PBS solution, embedded in OCT (Sakura Finetechnical) and then cryosectioned.

In situ hybridization

Whole-mount and slide in situ hybridizations were performed following established procedures (Jasoni et al., 1999; Schaeren-Wiemers and Gerfin-Moser, 1993). In situ hybridizations were carried out using antisense *Irx3, Six3, Gbx2, Dlx2* and *Wnt3* digoxigenin-labeled (Roche) riboprobes. Antisense riboprobes were prepared from plasmids containing chicken cDNA sequences for *Irx3*, a gift from Dr Jessell; *Six3*, a gift from Dr Shimamura; *Gbx2* (Kowenz-Leutz et al., 1997), a gift from Dr Leutz; and *Dlx2* (Puelles et al., 2000), a gift from Dr Rubenstein. Chicken *Wnt3* cDNA was cloned (C.P.R., M.M.B. and H.R., unpublished) and used to generate an antisense *Wnt3* digoxigenin-labeled riboprobe.

Headfold explants exposed to either Wnt3a, Dkk1 or cki7 were processed by in situ hybridization for *Irx3* and *Six3*. The expression domains of these markers were scored as normal, expanded and reduced/absent compared to headfold explants cultured under control conditions.

Prospective dT an vT explants were assayed in one of two ways post in situ hybridization. In both co-culture and Wnt3a-mediated *Gbx2* and *Dlx2* induction experiments, explants were scored as either positive or negative, as compared to the staining of control tissue. Prospective dT and vT explants cultured in supplemented neurobasal media served as the negative control. Tissue dissected from older embryos was used for both negative and positive controls.

In ovo manipulations

Electroporations were carried out following established protocols (Watanabe and Nakamura, 2000). *xDkk1* (Glinka et al., 1998), a gift from Dr Niehrs, was excised from *pCS2+* and cloned into *pMiwII* (Watanabe and Nakamura, 2000), a gift from Dr Nakamura. *eGFP* (Clontech) was cloned into *pcDNA3.1/Zeo* (Invitrogen). A mixture of 3 μg μl^{-1} *pMiwII-xDKK1* and 3 μg μl^{-1} *pcDNA3.1-GFP* in L-I5 (Gibco BRL) supplemented with 10 mM HEPES was injected into the neuropore of HH stage 9-10 embryos. Full-length chicken *Six3* (Kobayashi et al., 2002) was subcloned into *pMES-IRES-GFP* (Swartz et al., 2001). *pMES-Six3-IRES-GFP* resuspended in L-I5 (Gibco BRL) supplemented with 10 mM HEPES was injected into the neuropore of HH stage 9-10 embryos. The embryos were electroporated with two 25 msec pulses of 62.5 Volts cm^{-1}. After 48 hours, embryos were fixed and processed by in situ hybridization as described above.

Results

Wnt signaling confers posterior identity and inhibits anterior identity in HH stage-7 forebrain explants

By HH stage 7, the neural plate that overlies the prechordal plate expressed *Six3* and forebrain tissue overlying the anterior-most aspect of the notochord expressed *Irx3* (Fig. 1A,C). The adjacent, mutually exclusive expression patterns of *Six3* and *Irx3* confer regional identity on the early forebrain (Kobayashi et al., 2002). The interface between anterior and posterior forebrain overlies the transition between the prechordal plate and the notochord, and predicts the recognizable structure in the diencephalon.

To address the role that β-catenin-mediated Wnt signaling plays in the establishment and maintenance of *Six3* and *Irx3* in the early forebrain, we isolated headfold explants from HH stage 7-8 and cultured them for 24-48 hours in either the presence or absence of Wnt3a-conditioned medium (Shibamoto et al., 1998). The tissue was then fixed and assayed for *Six3* or *Irx3* expression by in situ hybridization. Untreated explants expressed *Six3* anteriorly and *Irx3* posteriorly in the explanted tissue (Fig. 2A,E). The addition of Wnt3a-conditioned supernatant resulted in a strong reduction or absence of *Six3* expression in all the treated explants (Fig. 2B). By contrast, the posterior expression pattern of *Irx3* following exposure to Wnt3a was not significantly different from control cultures (Fig. 2F) and an anterior expansion of *Irx3* expression was detected in at least a third of the explants (Fig. 2F), indicating that the loss of *Six3* can be followed by the induction of *Irx3* expression.

To address whether Wnt signaling was required for *Six3* or *Irx3* induction and maintenance, two independent, complementary approaches were taken. First, we used the pharmacological agent cki7 to inhibit the Wnt response in culture. This blocks the transforming Wnt pathway by repressing the phosphorylation of Dsh, a crucial step in the Wnt signaling cascade (Peters et al., 1999; Sakanaka et al., 1999). The addition of 50 μM cki7 (IC_{50}, 9.5 μM) (Chijiwa et al., 1989) was sufficient to reduce or eliminate the normal *Irx3* expression domain in 100% of the treated explants (Fig. 2G). Treatment with cki7 did not significantly expand the *Six3* expression domain (Fig. 2C).

To confirm the in vitro requirement for Wnt signaling on *Irx3* induction in posterior forebrain tissue, medium conditioned with *Xenopus* Dickkopf1 (Dkk1) was added to cultured headfolds. In *Xenopus*, Dkk1 antagonizes Wnt action (Glinka et al., 1998) by binding to LRP and Kremen (Mao et al., 2002; Nusse, 2001), members of a receptor complex for the Wnt ligand. Treatment with Dkk1 recapitulated the cki7 results, with 90% of the headfolds tested showing a downregulation of *Irx3* expression (Fig. 2H versus 2E). Again, the *Six3* expression pattern was unaffected by inhibition of the Wnt-signaling pathway (Fig. 2D).

These headfold explant experiments indicated that an early, endogenous Wnt signal was required for the maintenance of the posterior forebrain determinant *Irx3*. Moreover, if not inhibited, Wnt signaling can preclude the proper specification of the anterior forebrain by preventing the maintenance of *Six3* expression and the expansion of *Irx3* expression. To determine if this Wnt activity occurs in combination with other mesoderm-derived signals, we tested if neural plate explants exhibited a similar response.

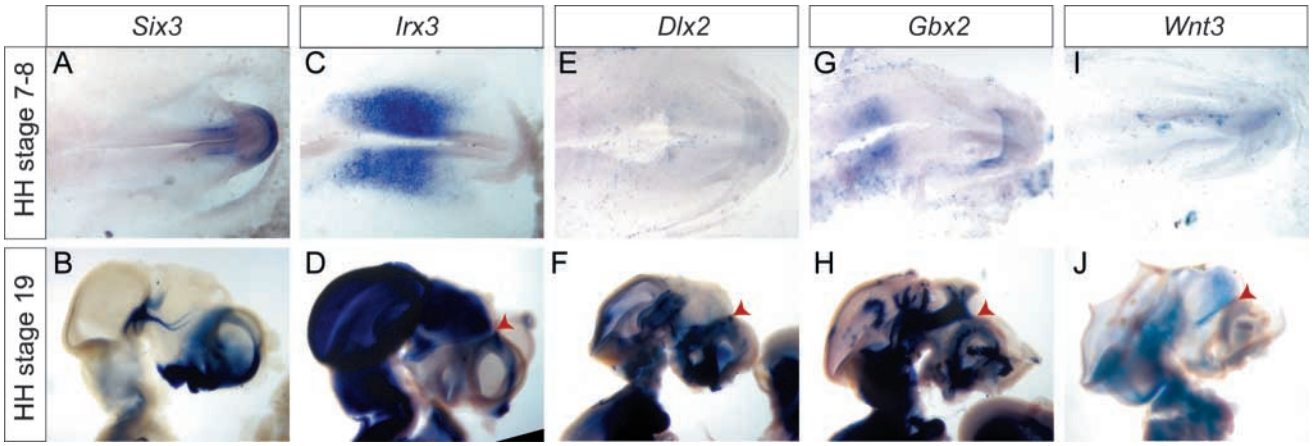

Fig. 1. *Six3*, *Irx3*, *Gbx2*, *Dlx2* and *Wnt3* expression in the developing forebrain. (A-J) In situ hybridization of *Six3* (A,B), *Irx3* (C,D), *Dlx2* (E,F), *Gbx2* (G,H) and *Wnt3* (I,J) at HH stage 7-8 (upper panels) and HH stage 19 (lower panels). At HH stage 7-8, *Six3* (A) and *Irx3* (C) are anterior and posterior to the prospective zli, respectively. At this stage, *Gbx2* (G) expression is confined to the prospective hindbrain, and *Dlx2* (E) and *Wnt3* (I) expression is negligible. At HH stage 19, the expression domains of *Irx3* (D), *Gbx2* (H) and *Wnt3* (J) are posterior to the zli, whereas *Six3* (B) and *Dlx2* (F) are expressed anterior to this structure. Arrowheads indicate the position of the zli. Scale bar: 100 μm.

Using the axial mesoderm transition as a guide, explants containing the prospective dT and vT were dissected from HH stage 8 chick embryos (Fig. 3A), cultured for 48 hours under serum-free conditions, fixed and then assayed for the induction of *Irx3*, *Gbx2* and *Dlx2* by in situ hybridization. Prospective dT explants, and not prospective vT explants, were *Irx3*-positive at the time of dissection (data not shown), consistent with the expression of *Irx3* in the posterior forebrain at HH stage 8.

Exposure to Wnt3a-conditioned medium increased the level of *Irx3* expression (Fig. 3F) and induced the expression of *Gbx2* (Fig. 3J), but only in dT explants. No significant induction of either *Irx3* or *Gbx2* was observed in vT explants (Fig. 3B). The anterior forebrain marker *Dlx2* was not expressed in dT and vT explants in the presence or absence of Wnts (Fig. 3K-N). These data demonstrate that Wnt signaling was sufficient to maintain posterior forebrain identity in explants. The Wnt-mediated induction of *Gbx2* indicates that a continuous Wnt signal is necessary for the development of the dorsal thalamus. Furthermore, the absence of Wnt does not cause expression of *Dlx2*, indicating that additional signals that are not provided in these in vitro cultures are required are required for its expression.

Blocking the response to forebrain-derived Wnt causes specific differentiation of the anterior forebrain

To test if zli tissue is capable of inducing vT- and dT-specific gene expression, prospective dT and vT explants from stage 8 embryos were cultured adjacent to zli explants from HH stage 17-18 embryos (Fig. 4A). Following 2 days in culture, induction of *Gbx2* and *Dlx2* was assayed by in situ hybridization. *Gbx2* was induced in 42% of the prospective dT explants cultured in contact with zli tissue, but in none of the prospective vT explants co-cultured with zli tissue (Fig. 4B,G,C). Co-culture of zli tissue with prospective vT explants

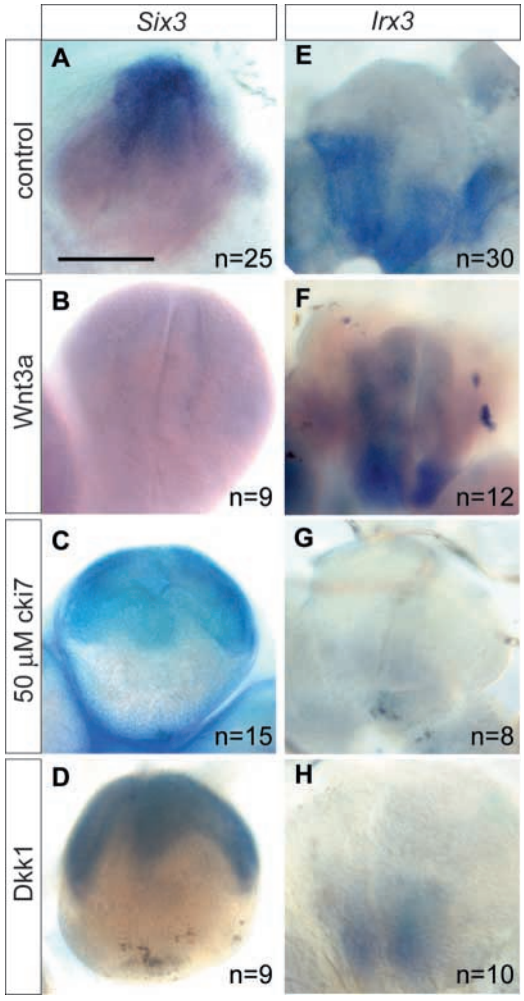

Fig. 2. Wnt signaling is sufficient to inhibit *Six3* expression and is required for *Irx3* expression in headfold explants. (A-H) Headfold explants were cultured in the presence or absence of Wnt3a, Dkk1 and cki7, and *Six3* or *Irx3* expression assayed by in situ hybridization. Representative headfold explants are shown following culture and in situ hybridization for *Six3* (A-D) or *Irx3* (E-H). *n*=the number of explants tested per condition. Scale bar: 100 μm.

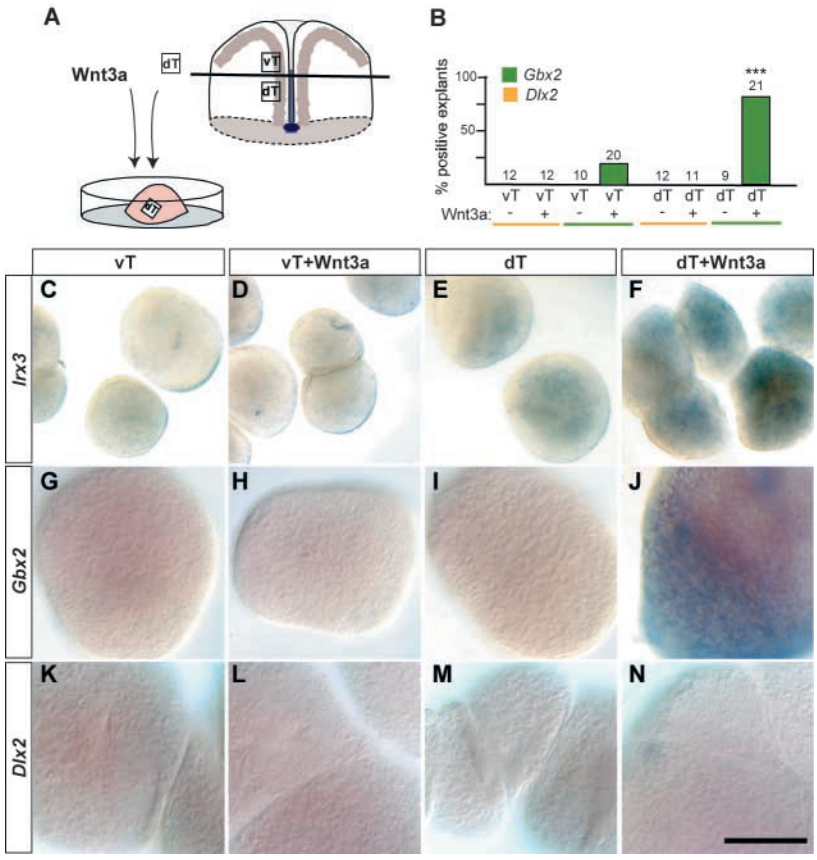

Fig. 3. Wnt3a induces *Irx3* and *Gbx2* in prospective dT explants. (A) The experimental procedure. Prospective vT and dT explants from HH stage 8 embryos were embedded in collagen, cultured in the presence or absence of soluble Wnt3a, fixed and assayed for *Irx3*, *Gbx2* and *Dlx2* expression by in situ hybridization. (B) The data was quantified and analyzed statistically using the χ-square significance test. (C-N) Representative explants in the culture condition indicated following in situ hybridization for *Irx3* (C-F), *Gbx2* (G-J) and *Dlx2* (K-N). Some panels show more than one explant. The total number of explants in each experimental condition are indicated above each bar in B. *** indicates $P<0.0001$. Scale bar: 250 μm in C-F, 100 μm in G-N.

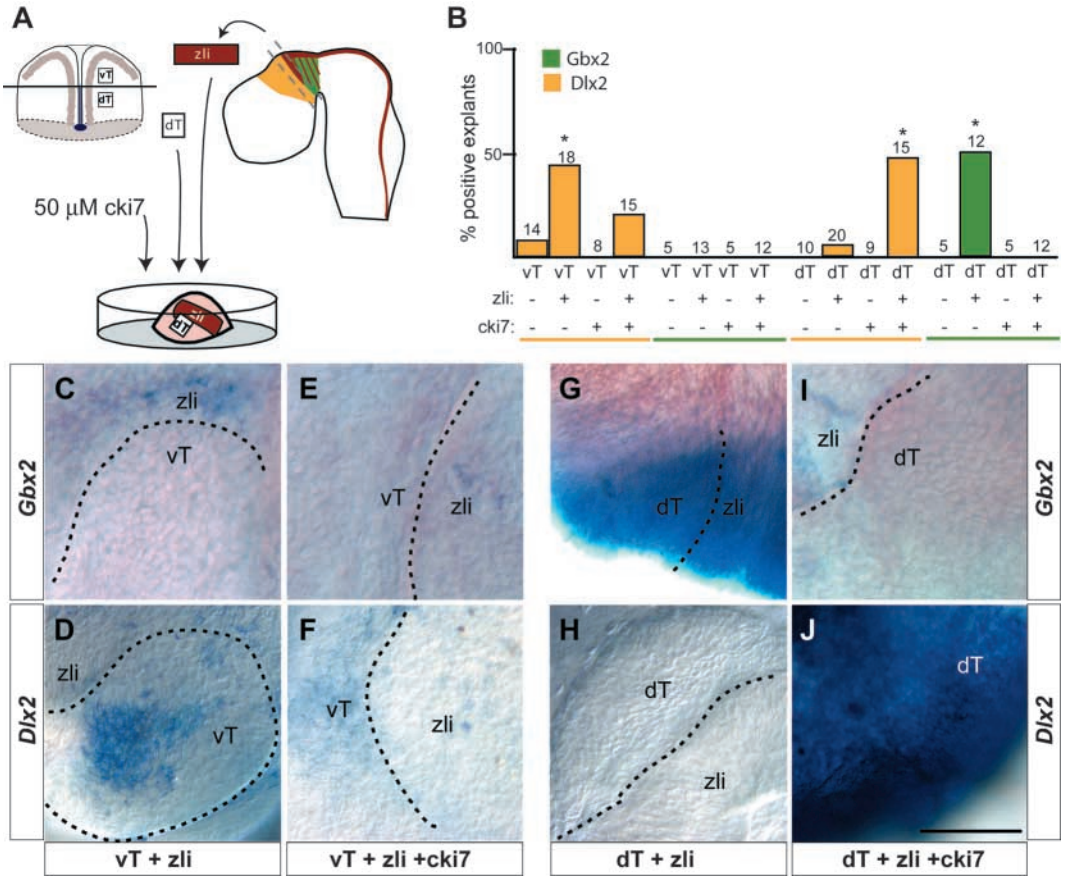

Fig. 4. Wnt signaling is required for dT-specific differentiation in response to zli-derived signals. (A) Diagram of experimental procedure. HH stage 17 zli explants were co-cultured with either prospective vT or dT explants from HH stage 8 embryos in the presence or absence of cki7 for 36-48 hours, fixed, and then processed by in situ hybridization. The horizontal line indicates the limit of the notochord and predicts the future location of the zli. (B) Co-culture induction data was quantified and analyzed statistically using a χ^2 significance test. (C-J) Representative in situ hybridization images for *Dlx2* (D,F,H,J) and *Gbx2* (C,E,G,I) in the indicated co-culture conditions. Dotted lines represent the border between prospective dT or vT explants and zli explants, as determined by using CellTracker. The total number of explants in each experimental condition are indicated above each bar in B. *, $P<0.05$. Scale bar: 100 μm.

induced *Dlx2* in 44% of these explants, whereas no significant *Dlx2* induction was observed in prospective dT explants cultured under these conditions (Fig. 4B,D,H). Because the zli explants are taken from HH stage 17-18 embryos, *Gbx2* and *Dlx2* expression are sometimes observed in zli tissue (e.g. Fig. 4C). These co-culture results confirm the observation that neural tissue anterior and posterior to the prospective zli has different competencies, which is consistent with the differential Wnt response in explants from these regions.

The addition of 50 μM cki7 altered zli-mediated inductive events in prospective dT explants. *Gbx2* induction was lost completely in prospective dT explants cultured with the zli and cki7 (Fig. 4B,I). Instead, the vT marker *Dlx2* was induced in 47% of prospective dT explants cultured in the presence of zli tissue and cki7 (Fig. 4B,J). Neither *Gbx2* nor *Dlx2* was induced by cki7 in prospective vT or dT explants that were cultured alone (Fig. 4B) and cki7 had no significant effect on the response of vT explants to zli-derived signals (Fig. 4B,F,E).

Together, these results demonstrate that, in vitro, a Wnt signal is required for both the induction of *Gbx2* and the repression of *Dlx2* in prospective dT explants. Apparently, blocking Wnt-mediated signaling causes an anterior to posterior change in the response to other signals derived from the zli. To address if blocking Wnt activity in the posterior forebrain allowed an anterior forebrain-specific response in ovo, we misexpressed *Xenopus Dickkopf1* (*xDkk1*). *pRK5-xDkk1* and *pCDNA3.1-GFP* were co-injected into the neuropore of HH stage 9-10 embryos and diencephalic misexpression of these plasmids was achieved following electroporation. Induction of *Dlx2* and *Gbx2* was visualized by in situ hybridization in serial sections in which GFP was present. Misexpression of *xDkk1* resulted in down regulation of *Gbx2* in dT tissue and a concomitant expression of *Dlx2* in the same region (Fig. 5C,D). The overlapping expression of these two markers was never observed in control embryos (Fig. 5A,B). Together, these results demonstrate that Wnt can be the sole determinant that allows posterior differentiation but that the induction of *Dlx2* requires other, unknown signals present in the zli or other parts of the forebrain. Because Wnt signaling efficiently represses *Six3* expression, and Six3 and Irx3 are mutually inhibitory, we tested if expression of *Six3* in the posterior forebrain resulted in differentiation appropriate for the anterior forebrain, as would be predicted based on a previous study (Kobayashi et al., 2002).

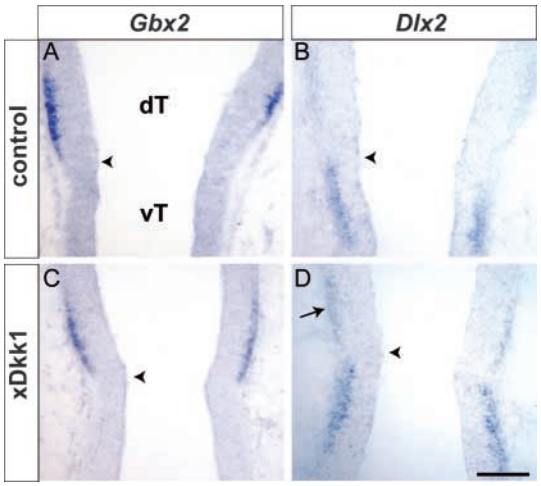

Fig. 5. Wnt is required in ovo for proper induction of *Gbx2* and repression of *Dlx2* in the diencephalon. (A-D) Adjacent sections were processed by in situ hybridization to visualize the expression of *Gbx2* (A,C) and *Dlx2* (B,D). Electroporation was used to misexpress *Dkk1* in the developing diencephalon of two HH stage-9 embryos (C,D). Ectopic expression of *Dkk1* resulted in ectopic expression of *Dlx2* (arrow in D) in the normally *Gbx2*-positive dT (A). Arrowheads indicate the position of the zli. Scale bar: 100 μm.

Six3 predicates vT-specific differentiation

Full-length chicken *Six3* (Kobayashi et al., 2002), cloned into *pMES-IRES-GFP*, was injected into the neuropore of HH stage 9-10 embryos, and diencephalic misexpression of this plasmid was achieved via electroporation. In serial sections where GFP was present posterior to the zli, *Dlx2* and *Gbx2* expression was visualized by in situ hybridization. Misexpression of *Six3* resulted in repression of *Gbx2* in dT tissue (Fig. 6A,B). In 25% of these embryos, a concomitant induction of *Dlx2* was observed in the dT (Fig. 6C). Independent electroporations demonstrated that misexpression of *Six3* in the posterior forebrain alters the normal levels of *Wnt3* in the dT; in regions of the dT where GFP was present, *Wnt3* was repressed (Fig. 6D,E,F).

These results indicate that *Six3* acts to specify late anterior forebrain differentiation, and is sufficient to allow both the induction of *Dlx2* and the repression of *Gbx2* and *Wnt3* expression. It remains to be determined if this effect is either direct or indirectly mediated by repression of *Irx3*.

Discussion

Wnt signaling in forebrain patterning

Combinatorial signaling is responsible for regional patterning in the CNS. However, the precise mechanisms by which the developing vertebrate forebrain is regionalized to give rise to structures such as the cortex, basal ganglia, thalamus and hypothalamus remain largely unclear. In this study, we investigated the role of Wnt signaling in establishing and maintaining regional identities in the developing diencephalon.

Wnt family members have been implicated as posteriorizing agents during neural development. In the Nieuwkoop model, neural induction occurs via a two-step activation-transformation process (Nieuwkoop, 1952). Following the initial induction of neural tissue, all of which is anterior in nature, subsequent events underlie the induction of more caudally-fated tissue. Wnts appear capable of mediating these secondary inductive events, thereby initiating posterior neural fates (McGrew et al., 1995). In *Xenopus*, misexpression of *xWnt8* results in loss of anterior structures, including the forebrain (Fredieu et al., 1997). Treatment with lithium, which activates the transforming Wnt pathway, has a similar effect.

In addition, the zebrafish mutant *mbl*$^{-/-}$, which has an over-active Wnt response caused by a nonfunctional *axin* gene, demonstrated a role for Wnt signaling in conferring posterior identity in the developing forebrain (van de Water et al., 2001). In *mbl*$^{-/-}$ mutants, there is a loss of telencephalon and vT with a concomitant expansion of the region that gives rise to dT. Because the telencephalon and vT develop from structures that are initially localized anterior to the dT, the fate shift in *mbl*$^{-/-}$ represents a gain of posterior-forebrain fates at the expense of anterior-forebrain fates. Although the exact identity of the Wnt ligands that mediate this posteriorization are unknown,

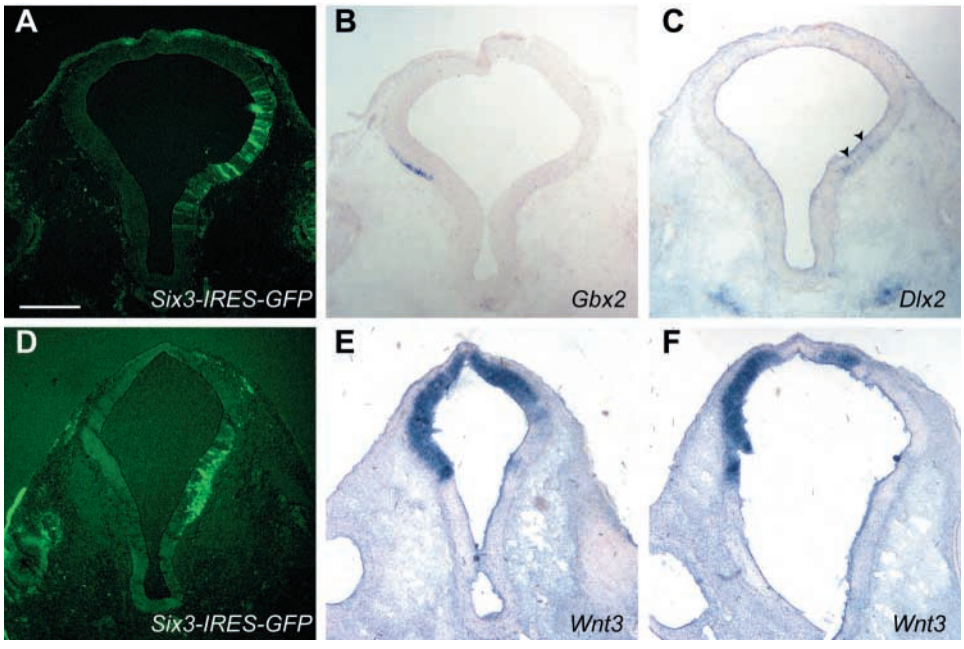

Fig. 6. In ovo misexpression of *Six3* suppressed diencephalic *Wnt3* and *Gbx2* expression and upregulated *Dlx2* in the dT. (A-F) Serial sections of embryos 48 hours after electroporation with *Six3-IRES-GFP* at HH stage 9-10. In sections in which GFP was visualized in the dT (A), *Gbx2* expression was suppressed (B, *n*=8). In 25% of these embryos *Dlx2* was induced ectopically in the dT (arrowheads in C). Misexpression of *Six3-IRES-GFP* in the dT (D) also results the down-regulation of *Wnt3* (E,F, *n*=2). Scale bar: 100 μm.

several candidate molecules are present in and around the developing forebrain during early and later stages of development (Nordstrom et al., 2002; Roelink and Nusse, 1991).

How are anterior and posterior forebrain competency differences established?

Although *Gbx2* and *Dlx2*, the markers of dT and vT forebrain fates, are not yet induced by HH stage 8, *Six3* and *Irx3* are expressed in distinct anterior- and posterior-forebrain domains at this time. The interface of *Six3* and *Irx3* expression corresponds with the boundary used to obtain prospective dT and vT explants: *Six3* is expressed above the prechordal plate and *Irx3* is expressed above the notochord. A study (Kobayashi et al., 2002) showed that the expression of either *Six3* anteriorly or *Irx3* posteriorly differentially primes anterior and posterior forebrain tissue to respond to Shh and Fgf signals, and results in the induction of either anterior forebrain or posterior forebrain-specific genes, respectively.

The Wnt inhibitor Dkk1 is produced in the prechordal plate and is present at the right time and place to block a tonic Wnt signal that confers posterior identity on the forebrain at neural-plate stages (Glinka et al., 1998). Moreover, Wnt family members are expressed in the posterior neural plate by HH stage 4-5 (Nordstrom et al., 2002), which indicates that Wnt signaling could be involved in early regionalization events of the developing forebrain. Our headfold culture experiments implicate differential Wnt signaling as the mechanism by which the forebrain determinants *Six3* and *Irx3* are induced in the neural plate. Ectopically supplied Wnt3a was sufficient to inhibit the expression of the anterior forebrain determinant *Six3*, whereas inhibition of the Wnt pathway eliminated the expression of the posterior forebrain determinant *Irx3*. These experiments support a model whereby Dkk1 from the prechordal plate inhibits a Wnt signal in the most anterior neural tissue, thus causing a switch from a Wnt-induced, *Irx3*-positive posterior forebrain fate to a *Six3*-positive, anterior forebrain fate (Fig. 7).

Prospective dT/vT explant culture experiments showed that these explants are not irreversibly committed to their appropriate anterior or posterior forebrain fates. Culture of headfold explants for 24 hours in the presence of Wnt-response inhibitors was insufficient to elicit a complete change from posterior to anterior identity, as measured by the expansion of *Six3* expression and the concomitant repression of *Irx3*. This indicates that respecification is a two-step process at least, in which the loss of Irx3 expression precedes the possible expansion of Six3. It might be predicted that extended culture in the presence of Wnt or Wnt-response inhibitors allows complete reprogramming of these explants to an alternate fate, but in these cases morphological changes in the explants precluded unambiguous interpretation of the in situ results. Nevertheless, our findings support a model whereby inhibition of the Wnt pathway in anterior tissue that overlies the prechordal plate represses posterior forebrain identity, and sets up an anterior fate on which later inductive cues can act (Fig. 7).

A Wnt signal is necessary and sufficient for specifying dT identity

Although the influence of Wnt signaling on *Irx3* expression indicates an early role for Wnts in forebrain patterning, the subsequent expression of *Wnt3* and *Wnt3a* indicate a subsequent role for Wnts in dT specification. An exogenous Wnt3/3a signal appears to be capable of acting as a posteriorizing agent that specifies the dT fate, as measured by *Gbx2* induction in our prospective forebrain-explant system. The observation that not all explants respond to Wnt3a-conditioned medium by expressing *Gbx2* indicates differences in these explants that might be caused by small variations between dissections and the embryonic stages from which these explants are derived. In addition, it remains to be determined whether higher doses of Wnt3a result in a higher percentage of *Gbx2*-expressing explants.

The ability of endogenous zli-derived signals to induce either vT or dT markers in prospective vT and dT explants was assessed using a heterochronic co-culture system. Because the expression patterns of inductive molecules at the zli are well-described at HH stage 17, zli tissue from these older embryos was used as a source of inductive molecules. Culture of zli tissue adjacent to prospective vT or dT explants is capable of inducing vT- and dT-specific gene induction. Because zli explants are not homogenous sources of inductive signals, we expect that the relative position of the prospective dT/vT explants to zli explants affects the induction of *Gbx2* and *Dlx2*,

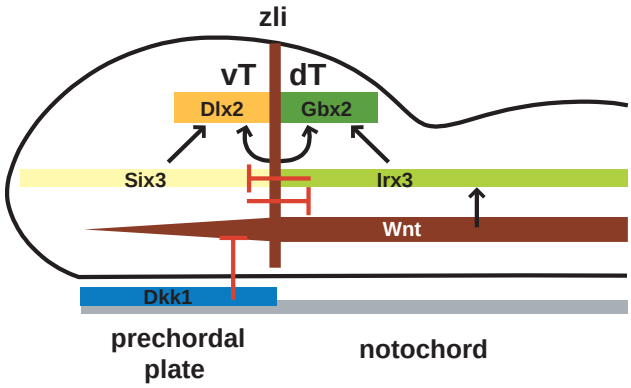

Fig. 7. Wnt signaling and diencephalic patterning. A widespread Wnt signal induces the expression of Irx3 in the developing forebrain, which specifies a posterior, dT-committed fate. Irx3 expression allows the induction of Gbx2 by signals released from the zli, which might include Wnt3 and Wnt3a. Neural tissue that overlies the prechordal plate is exposed to Wnt antagonists, which results in the expression of Six3. In turn, Six3 allows the induction of Dlx2 in response to zli-derived signals and represses the expression of Irx3, Wnt3 and Gbx2. This model predicts that the zli will form at the interface between the domains of Six3 and Irx3 expression, above the transition between the notochord and the prechordal plate. *Wnt3* is expressed throughout the prospective dT, just posterior to the zli (Fig. 1B) (Roelink and Nusse, 1991). The diencephalic phenotype of the *Wnt3a*-knockout mouse is unknown because it has a lethal gastrulation defect that prevents analysis of the role of *Wnt3* in forebrain development (Lee et al., 1997; Liu et al., 1999). Because of their largely overlapping expression patterns and nearly identical protein sequences, it is likely that Wnt3 and Wnt3a have partially redundant functions in brain development, which would explain the relatively mild phenotype observed in *Wnt3a*$^{-/-}$ mice. Given the expression patterns of *Wnt3* and *Wnt3a* in the diencephalon, these molecules are good candidates for inductive signals that either confer or maintain posterior identity on prospective dT tissue.

which might explain why just over 50% of the explants do not respond under these co-culture conditions.

The induction of *Gbx2* in prospective dT explants by zli tissue is mediated through a transforming Wnt signal, which is likely to be Wnt3 or Wnt3a. Inhibiting the Wnt response in co-cultures caused prospective dT tissue to acquire a vT-specific fate. Therefore, a Wnt signal that is either from the zli or present in the prospective dT is instructive in specifying the posterior/dT tissue in two ways. First, a Wnt signal induces the dT markers *Irx3* and *Gbx2*. Second, a Wnt signal inhibits the induction of the vT markers *Six3* and *Dlx2* (see Fig. 7). In turn, *Six3* inhibits the expression of *Gbx2* and *Wnt3* and promotes the expression of *Dlx2*.

Our observation that the vT is seemingly insensitive to Wnt signals could be caused by the lack of a crucial component of the Wnt signaling pathway in the vT. Interestingly, *Tcf4* is expressed in the prospective dT but not the vT (Galceran et al., 2000) and might be an important mediator of the differences in competency to respond to Wnt signaling on either side of the zli. Alternatively, Wnt inhibitors expressed in the prospective vT could prevent Wnt-receptor activation. SFRP-2, a known Wnt antagonist (Ladher et al., 2000), is expressed in the developing vT with a sharp posterior boundary of expression at the zli.

Although the in vitro studies presented in this work indicate a role for Wnt signaling in diencephalic development, they also demonstrate the existence of an undetermined factor or factors that induce the vT fate. Possible candidates for vT inducers include Shh and Fgf8. Both are expressed at the zli and we are currently examining their roles in *Dlx2* induction.

Our results agree fundamentally with the Nieuwkoop model of neural induction. However, the loss of anterior forebrain in Dkk1 mutants indicates that active suppression of Wnt signaling is required for the formation of anterior neural tissue, which implies the presence of tonic Wnt signals in the developing forebrain. It appears that the presence of Dkk1 is required for the expression of *Six3*, but it is unknown if *Six3* expression, in turn, requires a distinct inducer. Expression of *Irx3* in the posterior forebrain is likely to be induced by a Wnt signal, consistent with our observation that Wnt3a can induce *Irx3* in forebrain explants.

The expression of *Six3* and *Irx3* are crucial for the subsequent distinct differentiation of tissue in the anterior and posterior forebrain. Our observation that misexpression of *Six3* in the *Irx3* domain causes the repression of dT-specific and the activation of vT-specific gene expression demonstrates the key role of *Six3* in the induction of anterior forebrain fates. The normal pathway for vT specification, in which expression of *Six3* predicates that of *Dlx2*, involves continual inhibition of the Wnt response, whereas the inductive steps that allow *Gbx2* expression in *Irx3*-positive cells are mediated by Wnts, presumably Wnt3 and Wnt3a. The mechanism by which the interface of *Irx3* and *Six3* domains becomes the zli is unclear, but indicates the presence of signaling events at this border.

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