Development/Plasticity/Repair

Wnt Signal Specifies the Intrathalamic Limit and Its Organizer Properties by Regulating Shh Induction in the Alar Plate

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The structural complexity of the brain depends on precise molecular and cellular regulatory mechanisms orchestrated by regional morphogenetic organizers. The thalamic organizer is the zona limitans intrathalamica (ZLI), a transverse linear neuroepithelial domain in the alar plate of the diencephalon. Because of its production of Sonic hedgehog, ZLI acts as a morphogenetic signaling center. *Shh* is expressed early on in the prosencephalic basal plate and is then gradually activated dorsally within the ZLI. The anteroposterior positioning and the mechanism inducing *Shh* expression in ZLI cells are still partly unknown, being a subject of controversial interpretations. For instance, separate experimental results have suggested that juxtaposition of prechordal (rostral) and epichordal (caudal) neuroepithelium, anteroposterior encroachment of alar lunatic fringe (*L-fng*) expression, and/or basal Shh signaling is required for ZLI specification. Here we investigated a key role of Wnt signaling in the molecular regulation of ZLI positioning and *Shh* expression, using experimental embryology *in ovo* in the chick. Early *Wnt* expression in the ZLI regulates *Gli3* and *L-fng* to generate a permissive territory in which *Shh* is progressively induced by planar signals of the basal plate.

Introduction

The zona limitans intrathalamica (ZLI) is a neuroepithelial domain intercalated between the prethalamus and the thalamus in the alar plate of the diencephalon (prosomeres p3 and p2, respectively; Rendahl, 1924; Puelles et al., 1987; Rubenstein et al., 1994; Shimamura et al., 1995; Martínez and Puelles, 2000). The production and release of the Shh morphogen underlies the hypothesis that the ZLI is a secondary organizer. In addition to the ZLI, other neighboring diencephalic domains release signals such as retinoid acid or members of the Fgf, Bmp, and Wnt families. They jointly control the expression of regulatory genes encoding positional information in the thalamic neurepithelium, required to develop the diencephalic structural complexity (Scholpp and Lumsden, 2010; Martinez-Ferre and Martinez, 2012).

In chick embryos, it was proposed that interaction between prechordal [*Six3*-positive (*Six3*$^+$)] and epichordal (*Irx3*$^+$) neuroepithelium regulates ZLI specification (Kobayashi et al., 2002; Vieira et al., 2005; Guinazu et al., 2007). However, *Six3* is not required for the formation of the mammalian ZLI (Lavado et al., 2008). Alternatively, it also has been postulated that rostral *Fez* versus caudal *Otx* expressions regulates ZLI specification (Scholpp and Lumsden, 2010). The transverse ZLI is singular in brain regionalization, because it represents the only neural area in which Shh, normally a ventrodorsal polarizing signal (Ericson et al., 1996; Watanabe and Nakamura, 2000) regulates anteroposterior regionalization. However, the mechanisms underlying the positioning and activation of *Shh* in ZLI cells are still unclear. Interestingly, the intrathalamic boundary coincides with *Wnt8b* expression at early stages of development (Garda et al., 2002). This expression is complementary to the lunatic fringe (*L-fng*) expression domain, also held to be involved in ZLI formation (Zeltser et al., 2001).

Specification of cell identities in the neural tube is partly regulated by antagonistic interaction between dorsalizing and ventralizing signals, coded by Wnt (Lee and Jessell, 1999) and Shh (Jessell, 2000) signals stemming from the roof and floor plates, respectively. Such signals are reproduced and superposed at 90° in the alar diencephalon, in which *Wnt8b* is expressed transversally preceding *Shh* in the ZLI. Although some studies excluded a role of *Wnt* expression in *Shh* activation in the ZLI (Guinazu et al., 2007), we reexamined Wnt-related mechanisms controlling *Shh* expression in ZLI cells in chick embryos.

Our results show that inhibition of Wnt signaling in the alar diencephalon stops the dorsal progression of *Shh* expression in the ZLI through a Shh/Gli pathway-mediated mechanism. Gli3 is a transcriptional repressor regulated by Shh (Schimmang et al., 1992) and mediates Shh signaling (Persson et al., 2002; Abbasi et al., 2010). Our data indicate that Wnt-signal-mediated inhibition

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of *Gli3* precedes ZLI development and generates permissive conditions for the activation of *Shh* at the p3/p2 boundary. A broad initial expression of *Wnt8b* around the prospective organizer causes heterogeneous molecular interactions with other agents of ZLI formation, and the subsequent progressive reduction of the ZLI *Wnt* expression into a sharp transverse stripe may represent a mechanism organizing local positional information and stabilizing molecular interactions that underlie the specification of the morphogenetic organizer.

Materials and Methods

Experimental embryology

All animal experiments were performed in compliance with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC) and have been analyzed and approved by the Animal Experimentation Committee of our university. Fertilized chick (*Gallus gallus*) and quail (*Coturnix coturnix*) eggs were incubated at 37°C in a forced-air incubator. The embryos were staged according to Hamburger and Hamilton (1951).

Implantation of microbarriers

Metal microbarriers were implanted into the right side of the neural tube as described previously (Vieira and Martinez, 2006). Microbarriers were inserted between the basal plate and the prospective ZLI (Garcia-Lopez et al., 2004). Embryos were then allowed to develop until Hamburger–Hamilton stage 23 (HH23) before overnight fixation with 4% PFA in PBS at 4°C.

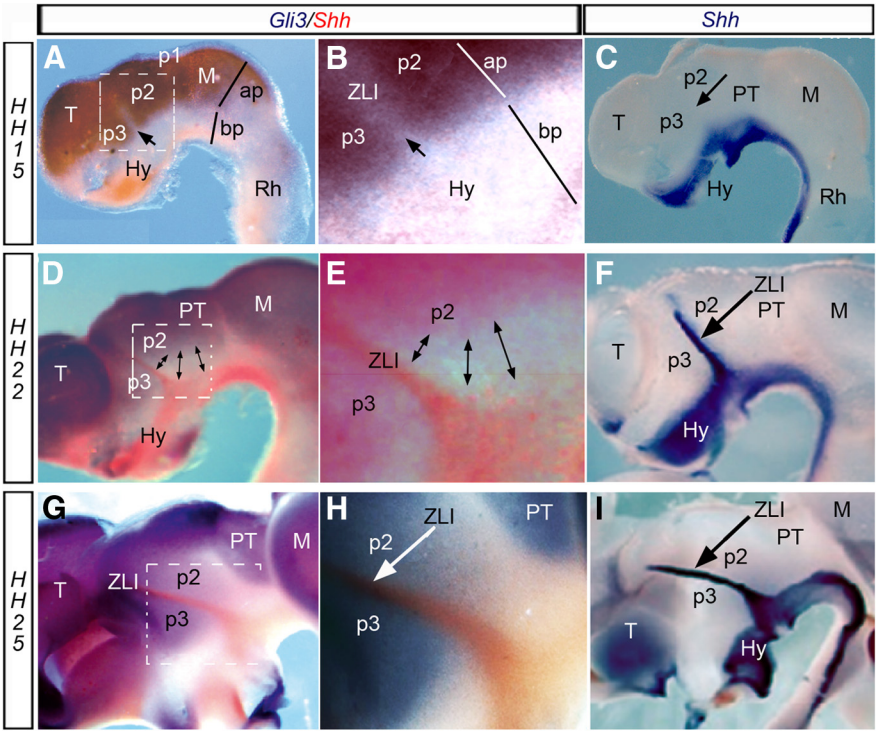

Figure 1. Lateral view of the gene expression pattern at different embryological stages. ***A–C***, HH15; ***D–F***, HH22; ***G–I***, HH25. ***A***,***B***, ***D***,***E***, and ***G***,***H*** show the complementary expression pattern of *Gli3* (blue) and *Shh* (red) in the chick diencephalon. ***B***, ***E***, ***H***, Higher-magnification of the squares represented in ***A***, ***D***, and ***G***, respectively. *Shh* expression (in blue) is analyzed at HH15 (***C***), HH22 (***F***), and HH25 (***I***), when *Fgf8* expression (red) is also detected. The arrow in ***A*** and ***B*** indicates the gap of *Gli3* expression in the presumptive ZLI at HH15. Arrow in ***C*** shows the lacking of *Shh* in the ZLI at this stage. Double-headed arrows in ***D*** and ***E*** indicate the absence of *Gli3* expression caudal to the ZLI (expressing *Shh*) at HH22. Arrowheads in ***C***, ***F***, ***H***, and ***I*** indicate the expression of *Shh* in the ZLI. ap, Alar plate; bp, basal plate; Hy, hypothalamus; M, mesencephalon; PT, pretectum; Rh, rhombencephalon; T, telencephalon.

Implantation of Dkk-1-soaked beads

Implantation of Dkk-1 beads into the neural tube of chick embryos was performed as described previously (Crossley et al., 1996; Vieira and Martinez 2005). Beads were implanted at the prospective ZLI at HH10. Heparin acrylic beads were rinsed in PBS and then soaked in a solution of 25 μg/ml Dkk-1 protein in PBS/0.1% BSA at 4°C overnight. Afterward, the beads were rinsed in PBS several times and then implanted into the neural tube of the embryos. For the control experiments, beads were soaked in PBS/0.1% BSA in the same manner. Embryos were fixed in 4% PFA at 4°C overnight, 24 h or 3–4 d after bead implantation.

Preparation of organotypic cultures

Chick embryos were developed until stage HH21 and prepared for organotypic culture as described previously (Echevarría et al., 2001). Briefly, chick neural tubes were dissected, telencephalic vesicles were partially removed, and neural tubes were opened through the dorsal midline and cultured ventricular side up. Implantation of beads was performed as described previously (Echevarría et al., 2001; Martinez-Ferre and Martinez, 2009). Dkk-1- or PBS-soaked beads were implanted at the dorsal end of the ZLI, visible at these stages using incident light in the central region of the diencephalic alar plate, and cultured for 24 h, before fixation.

Grafting experiments

Heterotopic and isochronic or heterochronic grafts were performed using quail embryos as the source of diencephalic or mesencephalic neuroepithelium implanted into the alar region of either the diencephalon (p1 or p2) or the mesencephalon of host chick embryos. The chimeric embryos were produced as described by Garcia-Lopez et al., (2004). After microsurgery, the eggs were sealed and incubated until stages HH23 or HH25.

In situ *hybridization*

After fixation, embryos were rinsed in PBT (PBS with 0.1% Tween 20), gradually dehydrated using increasing concentrations of methanol, and stored in 100% methanol at −20°C before being processed for in situ hybridization (ISH). Whole-mount ISH was performed as described previously (Shimamura et al., 1994). Digoxigenin and fluorescein-labeled RNA probes were prepared from plasmids from our laboratory collection or were kindly provided by the following laboratories: A. P. McMahon (Los Angeles, CA) (*Wnt8b*), C. Tabin (Boston, MA) (*Gli3*), and L. Puelles (Murcia, Spain) (*Gbx2*). ISH was performed for *Shh*, *Fgf8*, *Wnt8b*, *Wnt1*, *Otx2*, *L-fng*, *Gli3*, *Gbx2*, and *Nkx2.2*. RNA-labeled probes were detected with alkaline phosphatase-coupled anti-digoxigenin or anti-fluorescein antibodies (Roche Diagnostics). Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used as a chromogenic substrate to detect the digoxigenin-labeled probes (Roche Diagnostics), and iodonitrotetrazolium/5-bromo-4-chloro-3-indolyl phosphatase was used for the detection of the fluorescein-labeled probes (Roche Diagnostics). After ISH, embryos were washed in PBT, photographed under a dissecting microscope (Leica), and stored at 4°C in PBT with 0.1% sodium azide.

Analysis of the chimeras

After fixation, embryos were rinsed in PBT (PBS with 0.1% Tween 20), dehydrated using various concentrations of methanol, and stored in 100% methanol at −20°C before being processed for immunostaining. After processing for ISH, whole-mount immunostaining was performed using a monoclonal anti-quail antibody (QCPN; Developmental Hybridoma Bank) to detect quail tissues. Immunofluorescence for QCPN was performed by incubating the embryos with biotinylated goat anti-mouse antibody (1:200; Vector Laboratories). The secondary antibody was followed by incubation with streptavidin conjugated with Cy3 (1:700; GE Healthcare).

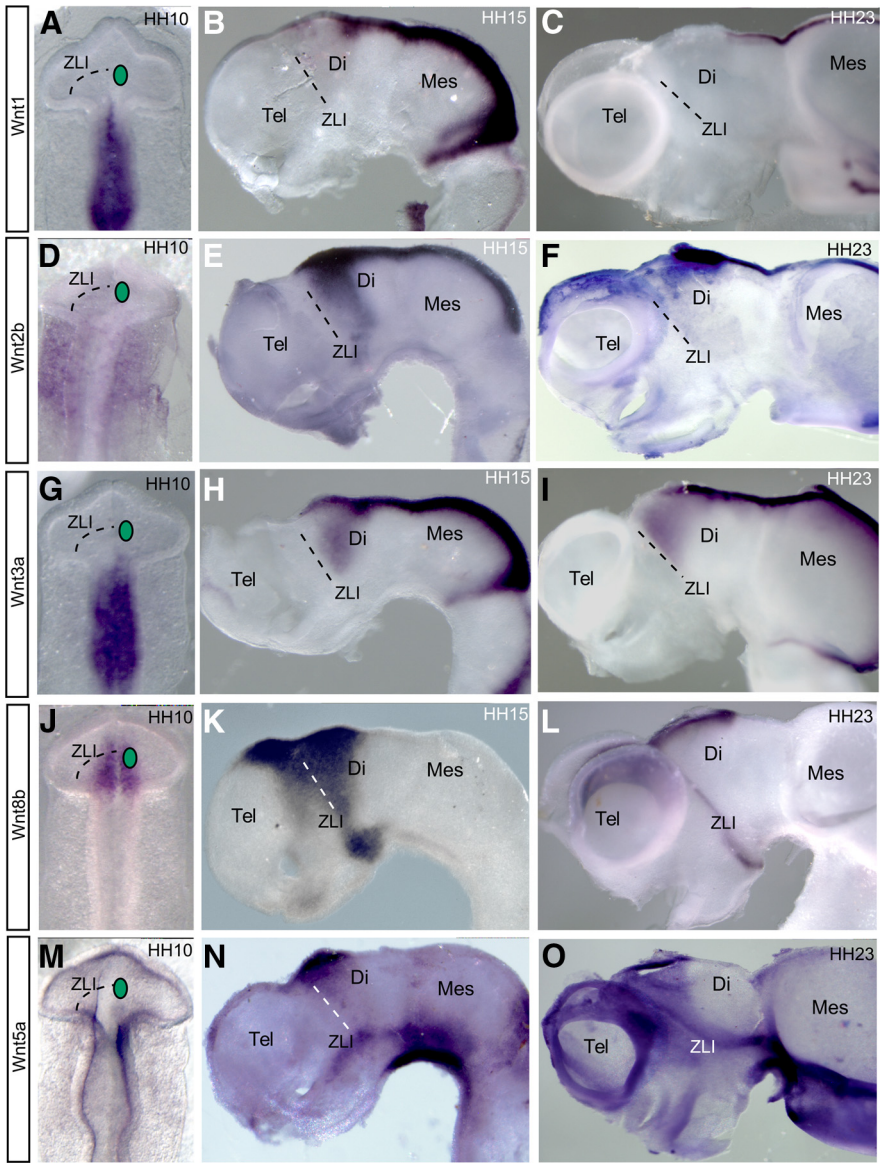

Figure 2. Wnt gene expression patterns in the chick prosencephalon: *Wnt1* (***A–C***), *Wnt2b* (***D–F***), *Wnt3a* (***G–I***), *Wnt8b* (***J–L***), and *Wnt5a* (***M–O***) at HH10 (***A***, ***D***, ***G***, ***J***, ***M***), HH15 (***B***, ***E***, ***H***, ***K***, ***N***), and HH23 (***C***, ***F***, ***I***, ***L***, ***O***). Dorsal views (***A***, ***D***, ***G***, ***J***, ***M***) and lateral views (***B***, ***C***, ***F***, ***E***, ***H***, ***I***, ***K***, ***L***, ***N***, ***O***) of whole-mount neural tube are shown. Dot lines mark the prospective ZLI. Green circles in ***A***, ***D***, ***G***, ***J***, and ***M*** indicate where a Dkk-1-soaked bead was implanted in bead-implanting experiments. Di, Diencephalon; Tel, telencephalon; Mes, mesencephalon.

WNT1-expressing cells

Cell culture. Cells from the QT-6 cell line were cultured in DMEM (Sigma), with 8% calf serum and 2% horse serum, supplemented with 100 U/ml penicillin–streptomycin (Sigma) and 2 mM L-glutamine (Sigma).

Mammalian expression vector. The mouse WNT1 coding region without stop codon (Clon IRAVp968F0412D; Imagenes) was subcloned into the *Bgl*II site of a pSTBlue1–mRFP1vector to obtain a WNT1–mRFP fusion protein. The cDNA fragment encoding mouse WNT1 and the mRFP1 coding region were subcloned into BamHI + NotI digested pIRES1hyg vector (Clontech) to generate pCMV–WNT1–mRFP1–IRES1hyg.

Transfection methods. One day before transfection (with Lipofectamine 2000; Invitrogen), the QT-6 cells were seeded at a density of 0.5×10^5 cells/cm^2 in multiwell (24-well) plates. The cells were incubated with DNA–lipid complexes for 4 h (following the instructions of the supplier), after which the lipofection mix was removed and replaced by fresh medium. Drug selection of stable transfectants was performed with 50–100 μg/ml hygromycin B (hyg; Calbiochem).

Graft of QT6–Wnt1 cells. Twenty-four hours before the graft, cells were seeded (1×10^6 cells/ml standard medium) into (35 mm) bacteria plates with 1% agar. Cell aggregates were transferred and grafted into chick embryos *in ovo*. Operated embryos were incubated at 37°C under humidified and ventilated atmosphere until they reached E3–E4 (HH20–HH23).

Results

Shh and *Gli* expression in the developing diencephalon

The Shh/Gli pathway plays a major role in dorsoventral patterning of the neural tube (for review, see Jessell, 2000; Ruiz i Altaba et al., 2007). In the diencephalon, Shh is the main signal expressed in the ZLI, a transverse morphogenetic organizer controlling anteroposterior diencephalic regionalization and thalamic nuclear organization in vertebrates (Echevarría et al., 2003; Vieira et al., 2005, 2010; Vieira and Martinez, 2006; for review, see Scholpp and Lumsden, 2010).

The first evidence of a ZLI in the alar diencephalic epithelium was detected at stage HH15, by the selective lack of alar *Gli3* expression between p2 and p3 (Fig. 1*A*,*B*). *Shh* signal did not appear at the ventral edge of this ZLI primordium until stage HH17 (Garcia-Lopez et al., 2004; Kiecker and Lumsden, 2004; Vieira et al., 2005). At later stages, *Shh* expression expanded dorsalward into the ZLI, filling the *Gli3*-negative locus between p2 and p3, and reaching its dorsalmost tip between stages HH22 and HH25 (Fig. 1*D–I*; Vieira et al., 2005).

Wnt expression in the developing diencephalon

Wnt/β-catenin activity apparently controls the expression of dorsal markers and suppresses the ventral program in the whole CNS (Backman et al., 2005; Alvarez-Medina et al., 2008). Because some *Wnt* gene expression domains correlate with morphological boundaries in the neural tube (Hollyday et al., 1995; Quinlan et al., 2009), we analyzed the temporo-spatial expression pattern of *Wnt1*, *Wnt2b*, *Wnt3a*, *Wnt8b*, and *Wnt5a* genes in the chick diencephalon (Fig. 2). Among these markers, *Wnt8b* expression appeared early in the diencephalon (HH10) and is the only member of the family expressed at the p3/p2 boundary before and after *Shh* activation (Fig. 2*J–L*). *Wnt2b* and *Wnt3a* are both expressed in an alar p2 region from HH15 onward but not at the ZLI (Fig. 2*D–F*, *G–I*, respectively); *Wnt7b* is expressed in rostral prosencephalic domains after HH22 (data not shown; Garda et al., 2002), whereas *Wnt5a* is expressed in p2 and p3 flanking the ZLI at later stages (HH23; Fig. 2*O*) and *Wnt1* is expressed at the dorsal midline of p1 and mesencephalon (Fig. 2*A–C*). Thus, although several *Wnt* genes expressed in neighboring regions generate Wnt signals acting on prospective diencephalic epithelium, their differ-

ent expression patterns at early postneurulation stages (HH9–HH11) strongly suggest that *Wnt8b* would be the favorite candidate to be involved in the specification of the p3/p2 boundary (the ZLI; Fig. 2). Moreover, *Wnt8b* expression appears dorsally in the area of contact between a graft of rostral forebrain neuroepithelium that is apposed to caudal alar forebrain in the diencephalic alar plate at HH10 [Garda et al., 2002; Garcia-Lopez et al., 2004; these were previously conceived as prechordal grafts into epichordal domains (Vieira et al., 2005), but the whole forebrain may be seen as being epichordal; Martinez et al., 2012; Puelles et al., 2012; Fig. 3*A*].

Later, at HH15–HH17, *Wnt8b* expression expands transversally ventralward along the p3/p2 limit, where it meets separate expression in the basal plate of p3 and hypothalamus; it also expands extensively along the diencephalic and telencephalic dorsal midline (Fig. 3*B*,*C*). At these stages, *Otx2* was homogeneously expressed in the diencephalic alar plate (Fig. 3*B*′). Later in development (HH23), *Wnt8b* expression remained strong in the telencephalic and diencephalic roof plate, whereas it was partially downregulated in the alar diencephalon, resulting in being restricted to the ZLI (Fig. 3*D*). *Wnt1* expression was mapped at the dorsal p1 midline and extends from there backward (Fig. 3*G–I*). At this stage, *Wnt8b* and *Shh* were coexpressed in the ZLI cells (Fig. 3*E*,*F*), flanked by prethalamic and thalamic neuroepithelial domains showing *Gli3* and *L-fng* expression (Fig. 1*B*,*C*,*E*,*F*; see Fig. 8*A*).

Thus, *Wnt8b* expression labels a central part of the prospective alar plate of the diencephalon at early neurula stage (HH10–HH14), and this expression becomes progressively restricted to the intrathalamic (p3/p2) limit between HH15 and HH17, preconfiguring the ZLI site before *Shh* expression appears (Figs. 1*C*, 2*K*, 3*B*). At stage HH23, *Wnt8b* becomes restricted to the ZLI, a part of the p3/p2 roof plate, and a narrow band that connects the basal end of the ZLI with the hypothalamic basal plate expression domain (Figs. 2*L*, 3*D*). This dynamic of the expression of *Wnt8b* attracted our interest to investigate the role of Wnt signals in the mechanisms regulating *Shh* expression in the ZLI and, subsequently, in the establishment of the ZLI organizer properties.

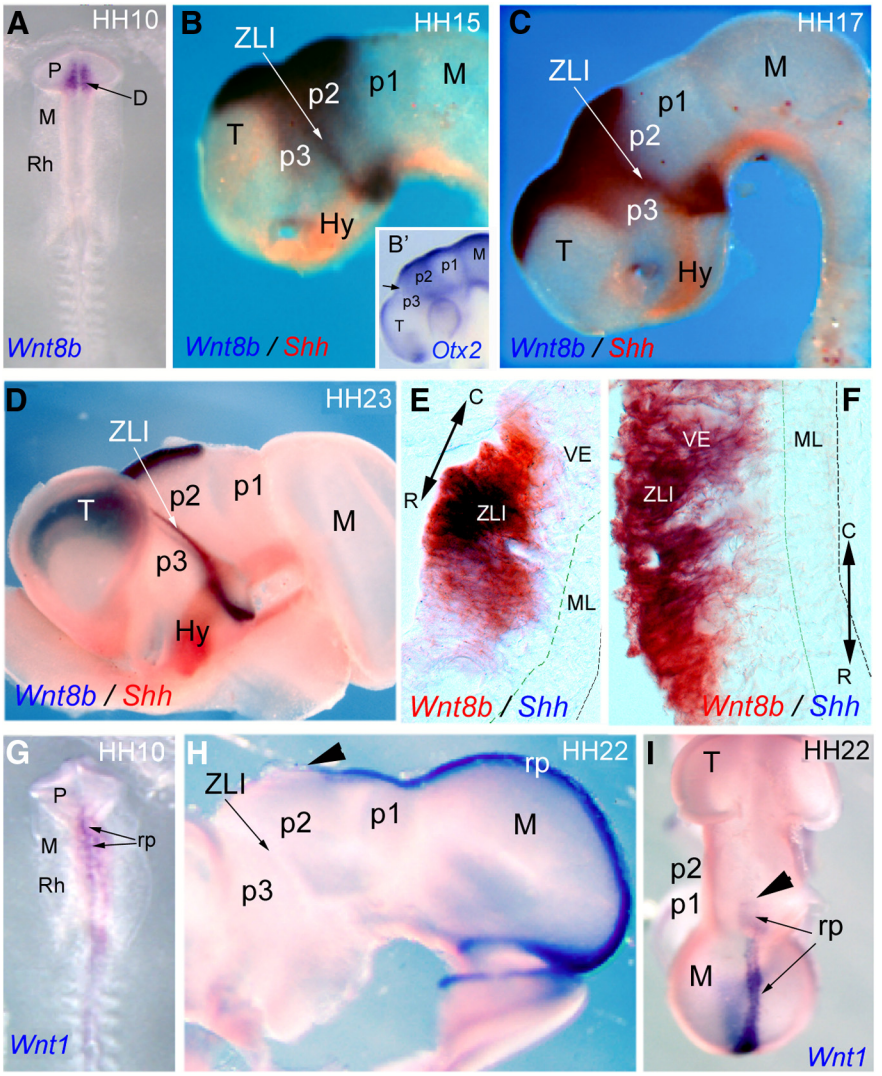

Figure 3. Analysis of *Wnt* expression at early stages of chick neural tube. ***A***, Dorsal view of an embryo analyzed by ISH for *Wnt8b* expression in the diencephalon (arrow) at stage HH10. ***B–D***, Lateral views of embryos analyzed by ISH for *Wnt8b* (blue) and *Shh* (red) at stages HH15, HH17, and HH23, respectively. ***B′***, Homogenous pattern of *Otx2* expression in the diencephalic alar plate at HH15. The arrow labels the position of ZLI. ***E***, ***F***, Cryostat horizontal sections (10 μm thick) of the ZLI (at the level indicated by the arrow tip) in ***C*** and ***D***. Embryos were analyzed by ISH for *Wnt8b* in red and *Shh* in blue. ***G–I***, Embryos analyzed by ISH for *Wnt1* at stages HH10 (***G***) and HH22 (***H***, ***I***). ***I***, Dorsal view of an embryo analyzed by ISH for *Wnt1* in the roof plate (arrows) at HH22. Arrowheads in ***H*** and ***I*** mark the rostral limit of *Wnt1* expression in the diencephalon. C, Caudal; D, diencephalon; Hy, hypothalamus; M, mesencephalon; ML, mantle layer; P, prosencephalon; R, rostral; Rh, rhombencephalon; rp, roof plate; T, telencephalon; VE, ventricular epithelium.

Signals from the basal plate do not affect *Wnt8b* expression in the ZLI

In a first approach, we inserted metal microbarriers in the primordial diencephalon, aiming to mechanically stop any upward cell movement in the prospective ZLI area, as well as any planar inductive interactions between alar and basal neuroepithelial cells, testing in such material the possible modification of *Wnt8b* expression in the ZLI (Vieira and Martinez, 2006). Microbarriers were inserted between the basal and alar plate at the prospective ZLI location in HH10 chick embryos (Fig. 4*A*), according to the diencephalic fate map (Garcia-Lopez et al., 2004). Embryos were collected at HH23 and examined to visualize the effect of the barriers on the expression of *Shh* and *Wnt8b* using double ISH (Fig. 4*B–D*).

Insertion of horizontal barriers was found to block *Shh* expression in the ZLI neuroepithelium dorsal to the barrier, as observed previously (Vieira and Martinez, 2006). However, we did not observe any effect on *Wnt8b* expression, either dorsally or ventrally to the barrier (Fig. 4*D–H*), except some directional anomalies attributable to scar formation in the epithelium around the barrier. Thus, although *Shh* was impeded from progressing dorsally into the diencephalic alar plate between stages HH17 and HH23, both *Wnt8b* expression (Fig. 4*B–F*; $n = 6$ of 7)

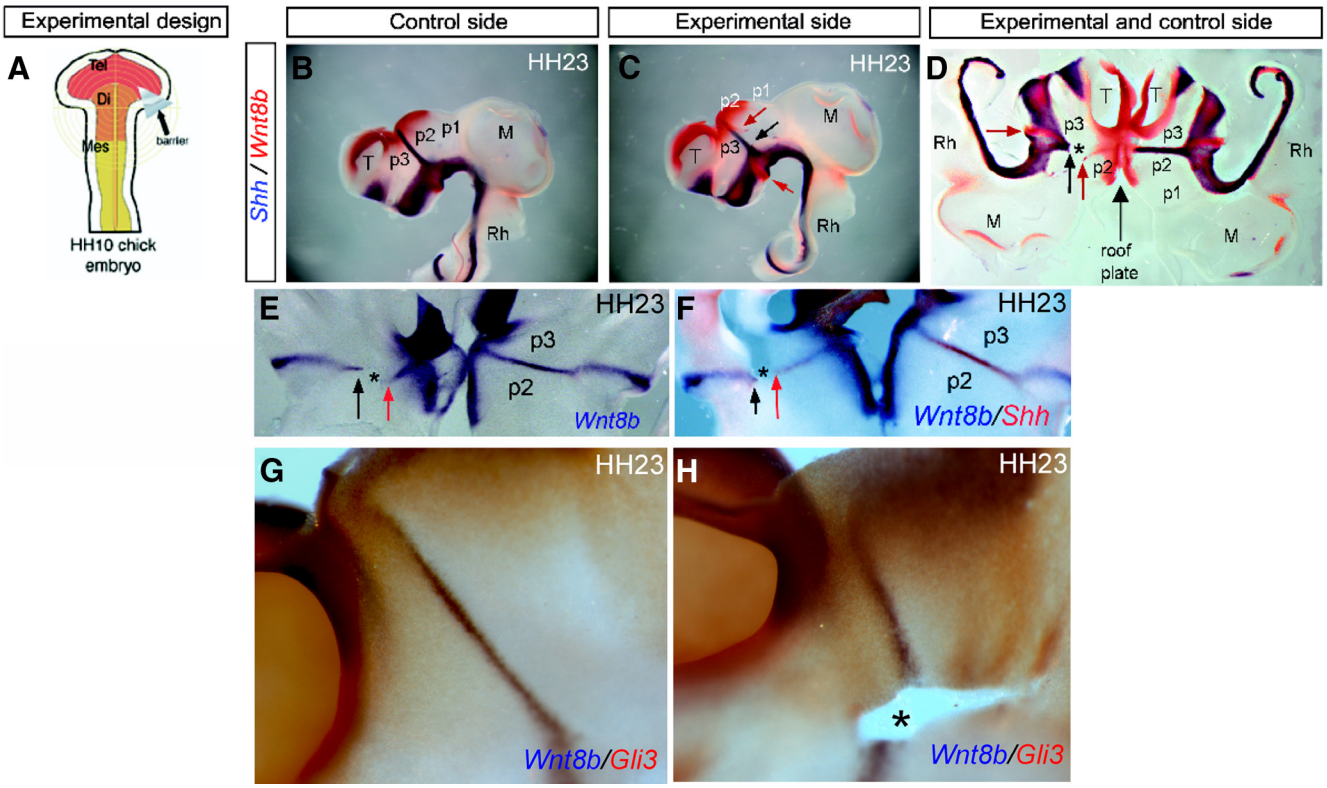

Figure 4. Implantation of microbarriers does not impede *Wnt8b* expression in the ZLI. ***A***, Schematic representation of barrier implantation in HH10 chick embryo. ***B***, Lateral view of the control side of HH23 chick embryos with double ISH for *Shh* in blue and *Wnt8b* in red. ***C***, Lateral view of the experimental side of HH23 chick embryos with double ISH for the indicated genes. Black arrow labels the level at which *Shh* expression ends in the experimental side; red arrows label *Wnt8b* expression in the basal and alar plates of the diencephalon. ***D–F***, Dorsal view of the embryos (opened through the ventral midline) analyzed by ISH for *Shh* in blue and *Wnt8b* in red (***D***; the same specimen as that in ***B***, ***C***), *Wnt8b* in blue (***E***), and *Shh* in red (***F***). ***C–F***, Black arrows indicate the dorsal limit of *Shh* expression in the experimental ZLI after microbarrier implantation. Red arrows indicate *Wnt8b* expression in the basal (caudal hypothalamus) and alar diencephalon (p3/p2 limit). ***G***, Lateral view of the control side of HH23 chick embryos with double ISH for *Wnt8b* in blue and *Gli3* in red. ***H***, Lateral view of the experimental side of HH23 chick embryos with double ISH for the indicated genes. Asterisks label microbarrier scars. Di, Diencephalon; M, mesencephalon; T, telencephalon; Rh, rhombencephalon.

and the gap lacking *Gli3* expression at the ZLI area were not modified (Fig. 4*G*,*H*; $n = 4$ of 4). Consequently, neither ventral influences, acting through planar induction, nor *Shh* expression in the incipient ZLI are necessary for *Wnt8b* expression and associated downregulation of *Gli3* at the incipient ZLI.

Inhibition of *Wnt8b* signaling by implanted Dkk-1 beads

To elucidate whether the observed Wnt signal has incidence on *Shh* expression in the ZLI, we next decided to block Wnt signaling by implanting Dkk-1-soaked beads in the diencephalic vesicle of HH10 chick embryos. PBS-soaked beads were used as control (Fig. 5*A*). Dkk-1 protein is a secreted inhibitor of canonical Wnt signaling, which modulates this pathway during embryonic development (Bafico et al., 2001; Mao et al., 2001). We focused our attention on *Wnt8b* expression because it is the only *Wnt* gene expressed in the diencephalic area of interest at the experimental stage (HH10). Our results showed that *Wnt8b* expression was indeed downregulated at the ZLI in the experimental Dkk-treated side in embryos fixed at HH15–HH16 (Fig. 5*B*,*C*; $n = 3$ of 4). Control experiments did not show any effect on *Wnt8b* expression (Fig. 5*D*,*E*; $n = 3$ of 3). As a control of tissue preservation, we performed double ISH to detect jointly *Fgf8* expression, which showed a normal expression pattern in the isthmus, dorsal diencephalon, hypothalamus, and septal telencephalon; this indicates that the experimental procedure did not cause collateral changes in neuroepithelial regionalization. According to these data, Wnt signals are required to maintain *Wnt8b* expression in the dorsal diencephalon; this suggests a positive autoregulatory (Wnt signaling) dependence mechanism.

Shh induction in the ZLI requires *Wnt8b* expression

Several studies have suggested that dorsal diencephalic tissue counteracts ZLI formation (Zeltser, 2005; Guinazu et al., 2007). The molecules secreted in the dorsal diencephalon include various members of the Bmp and Wnt families, as well as retinoic acid (Zeltser, 2005). These molecules hypothetically would inhibit *Shh* expression in this region. However, electroporation experiments using activators of the Wnt pathway do not inhibit *Shh* expression in the ZLI (Guinazu et al., 2007), suggesting that Wnt does not mediate the described inhibitory effect of dorsal diencephalic tissue. This is in agreement with our observations in which coexpression of *Wnt8b* and *Shh* was observed in ZLI epithelial cells (Fig. 3*D–F*). Furthermore, the expression of *Wnt8b* in the ZLI before *Shh* is activated also suggests that Wnt signals may rather play a positive role in this process. Therefore, we investigated whether Wnt signals generate a permissive territory for the ventrodorsal activation of *Shh* in the ZLI. Dkk-1 bead implantation experiments into the prospective ZLI at HH10 were analyzed after 4 d of postoperative incubation (Fig. 5*F*,*G*). Dkk-1 beads implanted in the prospective ZLI locus limited the dorsal progression of *Shh* expression at the ZLI (Fig. 5*F*,*G*; $n = 4$ of 5), whereas no effect was detected by inserting control (PBS-soaked) beads (Fig. 5*H*,*I*; $n = 3$ of 3). Therefore, Wnt signaling, probably represented by *Wnt8b* expression, is required in the diencephalon

for *Shh* activation in ZLI neuroepithelial cells. Insertion of Dkk-1 beads in different areas of prospective p2 and p1, to exclude additional Wnt inductive effects from more caudal regions, in which *Wnt1* and *Wnt3a* are later expressed (Fig. 2), did not modify *Shh* expression at the ZLI (see Fig. 8*G*,*H*; $n = 5$ of 5).

Next we studied whether the maintenance of *Shh* expression in the ZLI also requires Wnt signal. To assess this aspect, we prepared organotypic half-brain cultures from dissected chick neural tube at HH21 (Echevarría et al., 2001). Dkk-1 beads were inserted into the dorsal part of the ZLI, and the specimens were cultured for 24 h (Fig. 6). We observed that neither Dkk-1 nor PBS beads produced downregulation of *Shh* in the ZLI (Fig. 6*B*; $n = 8$ of 8). Thus, Wnt activity, despite being required for *Shh* activation in the ZLI epithelium, is not required for the maintenance of its expression.

Wnt signal controls *Gli3* expression by restricting spatially Shh/Gli activity

In agreement with Hashimoto-Torii et al. (2003), we observed that *Gli3* was differentially expressed in the developing alar diencephalon before *Shh* becomes expressed at the ZLI (Fig. 1*A*). In the HH15–HH16 chick diencephalon, a transverse alar stripe that is devoid of *Gli3* expression appears associated with the prospective locus of the ZLI expressing *Wnt8b* (Fig. 1*A*,*D*,*G*). We explored the possibility that Wnt signals may be downregulating *Gli3* expression at this locus, as is known to occur in the spinal cord (Alvarez-Medina et al., 2008). Therefore, we analyzed *Gli3* expression in the prospective ZLI after Dkk-1-bead implantation (Fig. 7*A*–*C*). This resulted in abnormal *Gli3* expression across the presumptive ZLI neuropithelium (Fig. 7*B*,*C*; $n = 7$ of 8). This result suggests that Wnt signaling is required for inhibition of *Gli3* expression in the prospective ZLI. The downstream repression of *Gli3* in the ZLI suggests a role of *Wnt8b* in establishing a permissive territory, which subsequently may allow the ventrodorsal progression of *Shh* expression. To test whether ectopic expression of *Wnt8b* generally causes *Gli3* repression and *Shh* induction, we grafted *Wnt8b*-expressing quail diencephalic neuroepithelium into the chick mesencephalon at HH10 (Fig. 7*D*). We describe here only grafts made into rostral mesencephalic areas because we found that *Wnt8b* expression in the donor tissue was not maintained if placed within the caudal diencephalon (data not shown). Normally, the mesencephalic alar

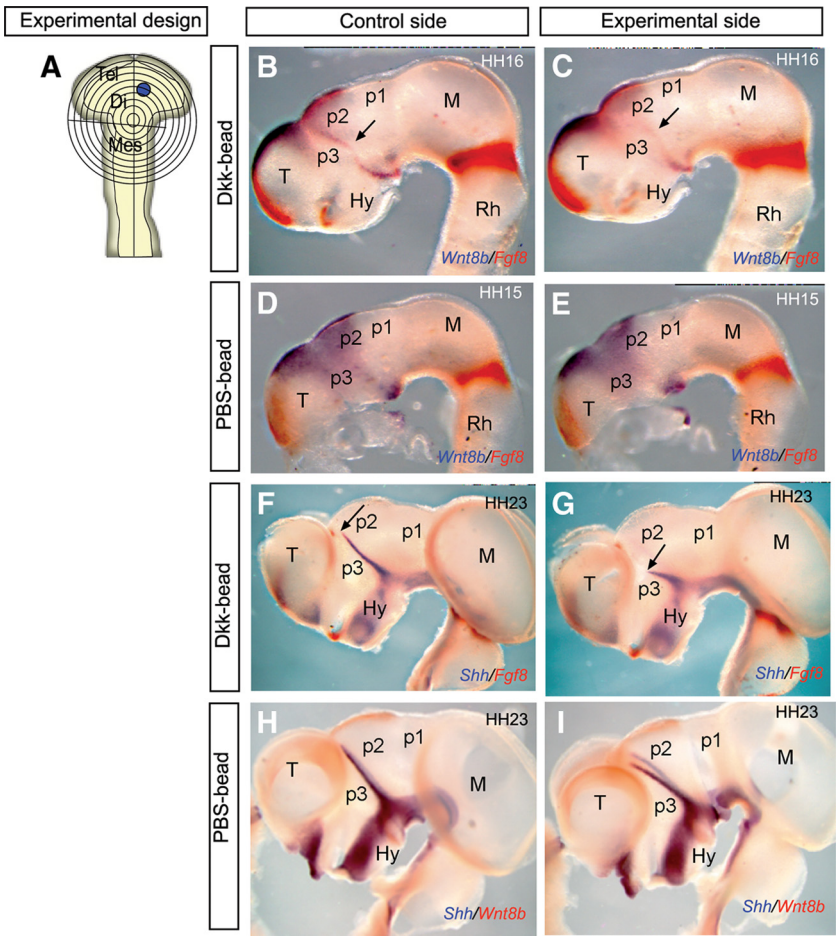

Figure 5. Inhibition of Wnt signaling blocks the dorsal progression of *Shh* expression in the ZLI. ***A***, Schematic representation of the bead-implanting procedure in which Dkk-1- and PBS-soaked beads were implanted into the prospective ZLI of chick embryos at HH10 (anterior pole of *Wnt8b* expression domain). ***B***, ***D***, ***F***, ***H***, Lateral views of the control sides of the chick embryos analyzed by double ISH for the indicated genes in each picture. ***C***, ***E***, ***G***, ***I***, Lateral view of the experimental side of chick embryos analyzed by double ISH for the indicated genes in each picture. ***B***, ***C***, Embryos implanted with Dkk-1-soaked beads and analyzed for *Wnt8b* and *Fgf8* expression 24 h after bead implantation. Arrows indicate *Wnt8b* expression in the ZLI (***B***) and its absence in the experimental side (***C***). ***D***, ***E***, Embryos implanted with PBS-soaked beads and analyzed for *Wnt8b* and *Fgf8* expression 24 h after bead implantation, showing similar expression of *Wnt8b* in the diencephalon. ***F***, ***G***, Embryos implanted with Dkk-1-soaked beads and analyzed for *Shh* and *Fgf8* expression 4 d after bead implantation. Arrow in ***F*** and ***G*** indicates the most dorsal level of *Shh* expression in the ZLI. Note the dorsal reduction of *Shh* expression in the experimental ZLI. ***H***, ***I***, Embryos implanted with PBS-soaked beads and analyzed for *Shh* and *Wnt8b* expression 4 d after bead implantation, showing normal expression of *Shh* in control and experimental sides. Di, Diencephalon; Hy, hypothalamus; M, Mes mesencephalon; Rh, rhombencephalon; T, Tel telencephalon.

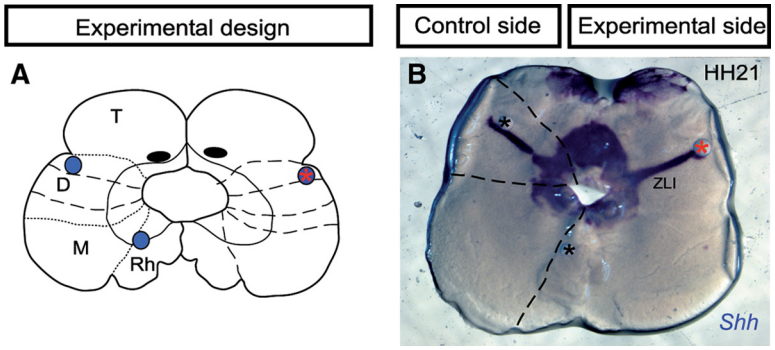

Figure 6. Wnt signaling is not required for the maintenance of *Shh* expression in the ZLI. ***A***, Schematic representation of the neural tube explants in which Dkk-1 or PBS-soaked beads were inserted at the left or right side, respectively. ***B***, *Shh* expression in HH21 cultured explants 24 h after culture. PBS-soaked beads (black asterisk) or Dkk-1-soaked beads (red asterisk) were inserted into the dorsal region of the ZLI of chick embryos at stage HH21. No modification of *Shh* expression was observed after blocking Wnt signaling in the experimental side. D, Diencephalon; M, mesencephalon; T, telencephalon; Rh, rhombencephalon.

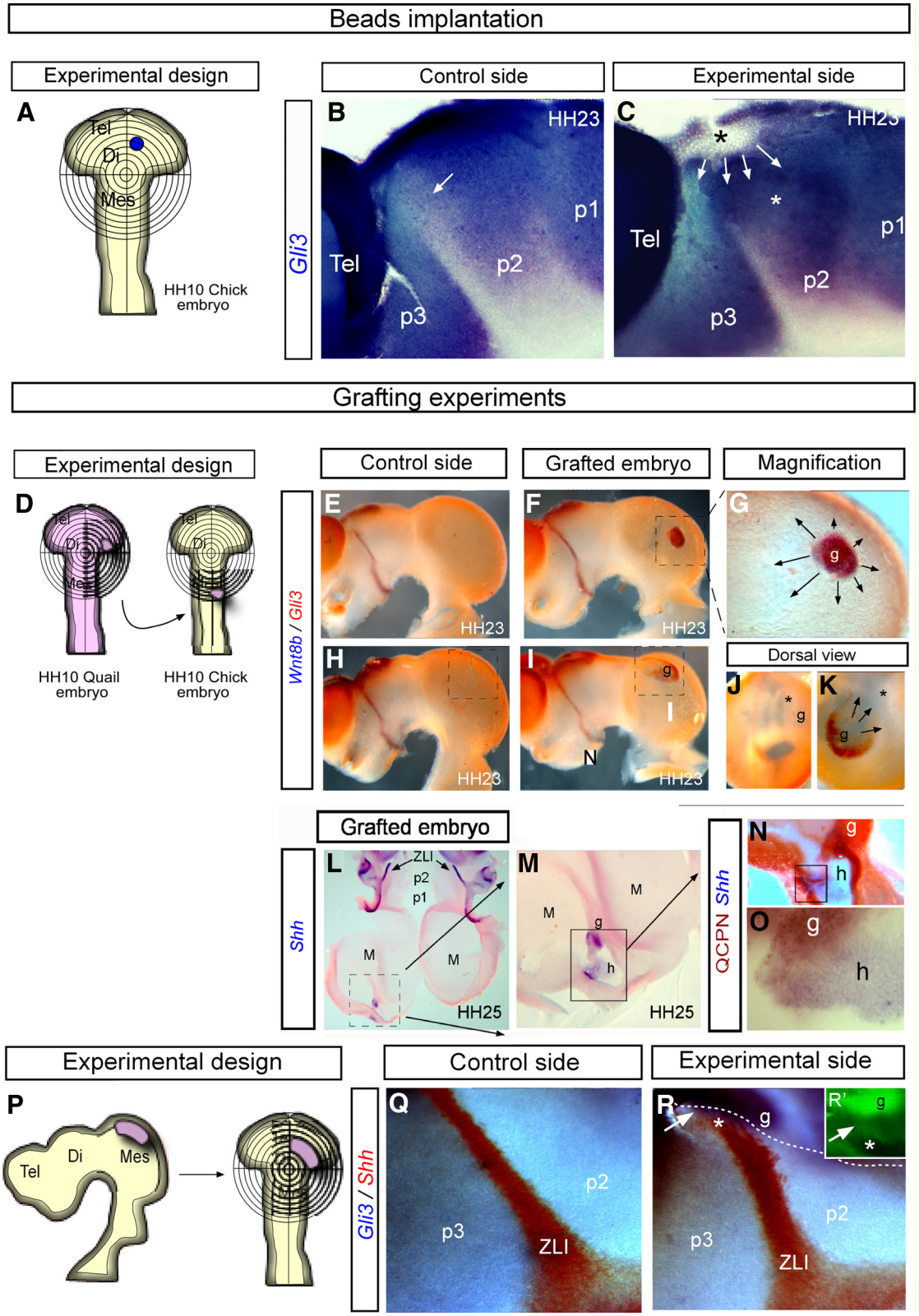

Figure 7. Wnt8b downregulates *Gli3* expression in the alar plate. ***A–C***, Inhibition of *Wnt8b* maintains *Gli3* expression in the prospective ZLI. ***A***, Schematic representation of the bead-implanting procedure in which Dkk-1- and PBS-soaked beads were implanted into the ZLI of chick embryos at HH10. ***B***, ***C***, Lateral views of the control side (***B***) and experimental side (***C***) of the chick embryos neural tube analyzed by ISH for *Gli3* in blue. Arrows in ***B*** localize the ZLI, in which *Gli3* is not expressed. In ***C***, *Gli3* is overexpressed in the diencephalic alar plate, making it impossible to distinguish the p3/p2 boundary in the most dorsal domain. ***D–O***, Grafting experiments. ***D***, Schematic representation of the grafting procedure in which *Wnt8b*-expressing neuroepithelium was transplanted from HH10 quail embryos into the alar plate of the mesencephalic vesicle of HH10 embryos. ***E***, ***H***, Lateral view of the control side of the neural tube at HH23 showing double ISH for *Wnt8b* (blue) and *Gli3* (red). ***F***, ***I***, Lateral views of the experimental side of the chimeric neural tube at HH23, analyzed by double ISH for *Wnt8b* (blue) and *Gli3* (red). ***G***, (*Figure legend continues.*)

plate homogenously expresses *Gli3* from stage HH15 onward (Fig. 1*A*,*D*,*G*). The operated embryos were allowed to develop for 2 d after the graft. We examined the chimeric embryos by ISH for *Gli3*, *Shh*, or *Wnt8b* and by immunohistochemistry for QCPN (Fig. 7*E–O*). Two effects were observed: *Gli3* expression was repressed around the grafts, which continued expressing *Wnt8b* (Fig. 7*E–K*), and *Shh* expression was induced in the neighboring host tissue (QCPN-negative epithelium) (Fig. 7*H–K*; $n = 5$ of 7). This result indicates that the transplanted tissue expressing *Wnt8b* (but not *Shh*) was capable of regulating in the host mesencephalon the expression of *Gli3* and *Shh*: repressing *Gli3* and activating *Shh*.

To explore whether *Gli3*-expressing neuroepithelium was sufficient to stop upward *Shh* induction in the primordial ZLI, we grafted alar mesencephalon of HH23 quail embryos into the dorsal p2 region of chick embryos at HH10 stage (Fig. 7*P*). After 3 d of survival, we observed continued expression of *Gli3* and a failure of *Shh* induction in the graft-derived area of chimeric embryos (Fig. 7*R*; 5 of 5). Moreover, a narrow epithelial band of the host diencephalic epithelium was induced to express *Gli3* at the graft/host boundary; these host cells also did not activate *Shh* expression (Fig. 7*R*,*R′*, white arrow; $n = 3$ of 5). Undoubtedly, the replacement of the *Wnt8b*-expressing intrathalamic domain by *Wnt8b*-negative donor mesencephalic epithelium eliminated required *Wnt* signals and, consequently, *Gli3* expression is maintained at the grafted mesencephalon and neighboring host epithelium (Fig. 7*P–R*). The possibility of an experimental artifact is discarded, because inductive planar interactions between quail and chick neuroepithelial cells have been corroborated extensively in the diencephalon (Vieira and Martinez, 2006), and chimeras carrying diencephalic homotopic grafts develop normally (Garcia-Lopez et al., 2004).

Wnt8b defines *L-fng* expression domain

We decided to further analyze whether other molecules that control *Shh* progression in the ZLI may be also under the control of Wnt signaling. *L-fng* is expressed in the diencephalon flanking the ZLI once this is formed and is normally dynamically complementary to *Wnt8b* expression in the diencephalon, expanding as the latter becomes restricted at the ZLI, thus progressively encroaching on the latter (Garcia-Lopez et al., 2004). The molecular and cellular mechanisms regulating this dynamic *L-fng* expression pattern are still unknown. Moreover, previous studies showed that *L-fng* can repress *Shh* expression at the ZLI (Zeltser et al., 2001). To determine whether Wnt signals influence *L-fng* expression, we inserted Dkk-1- and PBS-soaked beads into the prospective ZLI of chick embryos at HH10 and analyzed *L-fng* and *Shh* expression 4 d after bead implantation (Fig. 8). We observed that the encroachment of the *L-fng*-expressing domain on the dorsal part of the ZLI was impeded in the experimental side, associated with a lack of *Shh* induction at the dorsal tip of the ZLI (Fig. 8*A–F*; $n = 10$ of 13). When a Dkk-1 bead was implanted in the caudal diencephalon (counteracting other potential Wnt signals), the encroachment of *L-fng* around *Shh* expression in the ZLI was not abolished (Fig. 8*G*,*H*; $n = 4$ of 4). Conversely, when only *Shh* expression at the ZLI was abolished by insertion of a horizontal barrier, a normal *L-fng* expression pattern was observed, suggesting that the encroaching *L-fng* expression is regulated by the signal present at the p2/p3 limit and not by secondary Shh signal (Fig. 8*I–K*; $n = 4$ of 5).

The p3/p2 boundary itself is regulated by Wnt signaling

It is known that Shh acts in the ZLI as a signaling molecule that regulates the expression of developmental genes that will specify compartmentalization of neighboring diencephalic progenitor areas (Echevarría et al., 2003; Vieira et al., 2005), such as domains expressing *Gbx2* or *Nkx2.2*. *Gbx2* is a homeobox gene known to be expressed in thalamic mantle layer cells, and some studies have demonstrated a positive regulation of *Gbx2* by Shh (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira and Martinez, 2006). Moreover, *Nkx2.2* is a homeodomain transcription factor (TF) expressed in the diencephalon separating the basal and alar plates (Shimamura et al., 1995) and also flanking the ulterior expression of *Shh* in the ZLI (Martínez-de-la-Torre et al., 2002). As expected (Vieira et al., 2005), when we implanted Dkk-1-soaked beads in the dorsal diencephalon and observed a truncation of ZLI *Shh* induction (Figs. 5*C*, 8*E*), there also appeared a dorsal zone with corresponding reduction of *Gbx2* and *Nkx2.2* expression (Fig. 9, black arrowheads; $n = 5$ of 8).

Surprisingly, the experimental side of the embryos receiving Dkk-1 beads often showed an abnormal expression of *Nkx2.2* and *Gbx2* covering the dorsal tip of the stunted ZLI (Fig. 9, white arrowheads; $n = 5$ of 8), forming a bridge between p2 and p3; clearly the p3/p2 boundary in not established in the absence of Wnt signal.

An ectopic source of Wnt signal induces molecular reorganization in the host tissue

Our experiments strongly suggested that a Wnt signal is required as first permissive step for the subsequent activation of *Shh* expression in the ZLI; this effect is thought to occur via locally restricted negative regulation of *Gli3* expression at the center of the early Wnt8b expression domain. To confirm this hypothesis, we implanted *Wnt1*-expressing cells in the dorsal diencephalon of chick embryos at HH10 (we do not have cells expressing *Wnt8b*; it was hoped that other Wnts signals can duplicate the postulated *Wnt8b* function). After 3 d of incubation, we explored the effect of supposedly physiological levels of ectopic Wnt1 signal on *Gli3* and *Shh* expression. In the dorsal diencephalon of the experimental side, we observed a reduction of the domain of *Gli3* expression in the dorsal thalamus (Fig. 10, pink arrowheads; $n = 5$ of 7), together with an ectopic expression of *Shh* in this area (Fig. 10, black arrowheads; $n = 5$ of 7).

Thus, Wnt signals operating in the ZLI seem to regulate both the early downregulation of *Gli3*, which allows ventrodorsal homeiotic *Shh* induction from underlying basal *Shh*-positive domain, and parallel encroachment of *L-fng* on the forming ZLI (Fig. 11). This Wnt activity is held to be mediated normally by *Wnt8b*, because it is the main Wnt gene expressed at the ZLI before *Shh* is expressed in that area.

←

(*Figure legend continued.*) Higher-magnification view of the square represented in ***F***. ***J***, ***K***, Dorsal view of control (***J***) and grafted (***K***) embryos, showing the expression of *Gli3* in the mesencephalon and its repression around the *Wnt8b* graft. ***L–O***, The host in which *Gli3* was downregulated activated *Shh* expression. ***L***, Flat mount of the chimeric neural tube, opened through the ventral midline and analyzed for *Shh* by ISH. ***M***, ***N***, Higher-magnification view of the square represented in ***L***. ***M***, Shows *Shh* ectopic activation in the mesencephalic alar plate. ***N***, ***O***, Higher-magnification views of the same embryo shown in ***L***, analyzed by ISH for *Shh* in the QCPN-negative (host) territory. Quail cells (maroon) do not expressed *Shh* (blue). ***P***, Schematic representation of the grafting procedure in which *Gli3*-expressing neuroepithelium was transplanted from HH23 quail embryos into the alar plate of the diencephalic vesicle of HH10 embryos. ***Q–R***, Lateral views of the control side (***Q***) and experimental side (***R***) of the chick embryos neural tube analyzed by double ISH for *Gli3* in blue and *Shh* in red; ***R′***, QCPN $^+$ cells were detected by immunofluorescence. White arrows in ***R*** and ***R′*** localize host cells expressing *Gli3*. Di, Diencephalon; g, graft tissue; h, host tissue; M, Mes, mesencephalon; Tel, telencephalon.

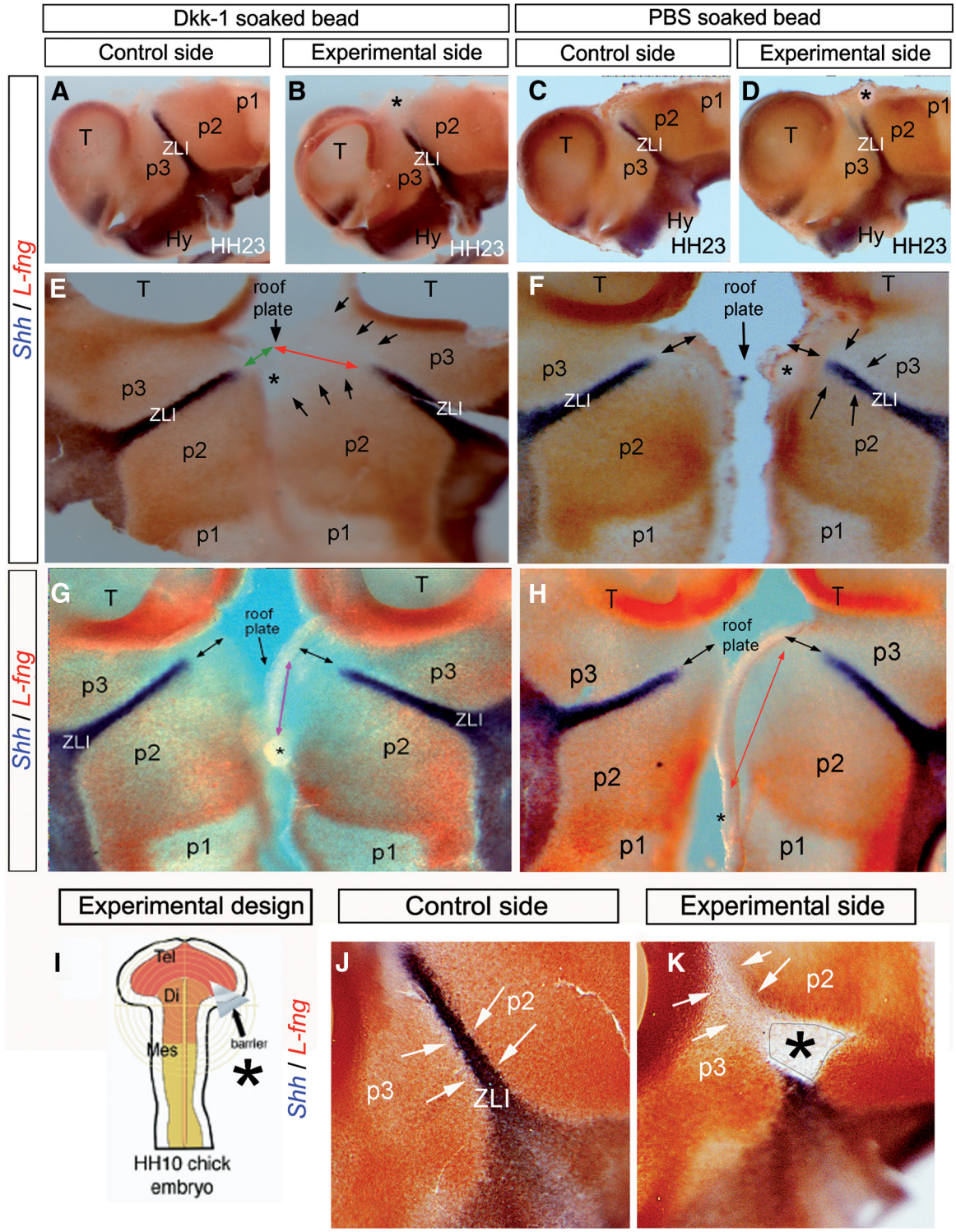

Figure 8. Wnt signaling is required for *L-fng* encroachment in the alar diencephalon. ***A–D***, ***J***, ***K***, Lateral views of the whole-mount prosencephalic neural tube stained for *Shh* (blue) and *L-fng* (red) by double ISH at HH23. ***A***, ***C***, ***J***, Control side of the experimental embryos. ***B***, ***D***, ***K***, Experimental side of the embryos. ***E–H***, Flat mounts opened through the ventral midline. Asterisk in ***B***, ***E***, and ***G*** indicates the region in which a Dkk-1-soaked bead was implanted at HH10. Asterisk in ***D***, ***F***, and ***H*** indicates the region in which the PBS-soaked bead was implanted. Green and red arrows in ***E*** indicate the distance between the dorsal midline and the dorsal level of *Shh* expression in the control (green) and experimental (red) side of an embryo in which a Dkk-soaked bead was implanted (asterisks). Black arrows in ***E*** indicate that *L-fng* encroachment was disrupted in the experimental side of the embryo. Black arrows in ***F*** and ***H*** indicate the similar distance between the dorsal midline (roof plate) and the dorsal level of *Shh* expression in the control and experimental side of an embryo in which the PBS-soaked bead was implanted. Black arrows in ***G*** indicate the same distance between the dorsal midline (roof plate) and the dorsal level of *Shh* expression in the control and experimental side of an embryo in which the Dkk-1-soaked bead was implanted in the most caudal position of the alar/roof plate of p2. ***I***, Schematic representation of barrier implantation in HH10 chick embryo. ***J***, Lateral view of the control side of HH23 chick embryos analyzed by double ISH for *Shh* (blue) and *L-fng* (red). ***K***, Lateral view of the experimental side of chick embryos. Asterisk in ***K*** indicates the region in which the microbarrier was implanted at HH10. Arrows in ***J*** and ***K*** indicate that the expression of *L-fng* at both sides of the ZLI was not disrupted in embryos operated with the microbarrier, in which *Shh* was blocked to progress through the ZLI. Di, Diencephalon; Hy, hypothalamus; Mes, mesencephalon; T, Tel, telencephalon.

Discussion

The ZLI is an important diencephalic organizer (for review, see Scholpp and Lumsden, 2010; Martinez-Ferre and Martinez, 2012). Apart from the gradiental distribution of Shh spreading from the ZLI and the underlying basal plate, relevant positional information is provided also by other signaling molecules, such as Fgfs (Kataoka and Shimogori, 2008; Martinez-Ferre and Martinez, 2009), Bmp (Furuta et al., 1997; Lim et al., 2005), and Wnts (Braun et al., 2003; Zhou et al., 2004; Bluske et al., 2009). These jointly modulate the regional expression of various TFs, whose downstream effects regulate proliferation (Juraver-Geslin et al., 2011) and cell differentiation in the prethalamus and thalamus. The singularity of the ZLI, as a transversal spike apparently arising from the longitudinal basal plate, has long attracted attention.

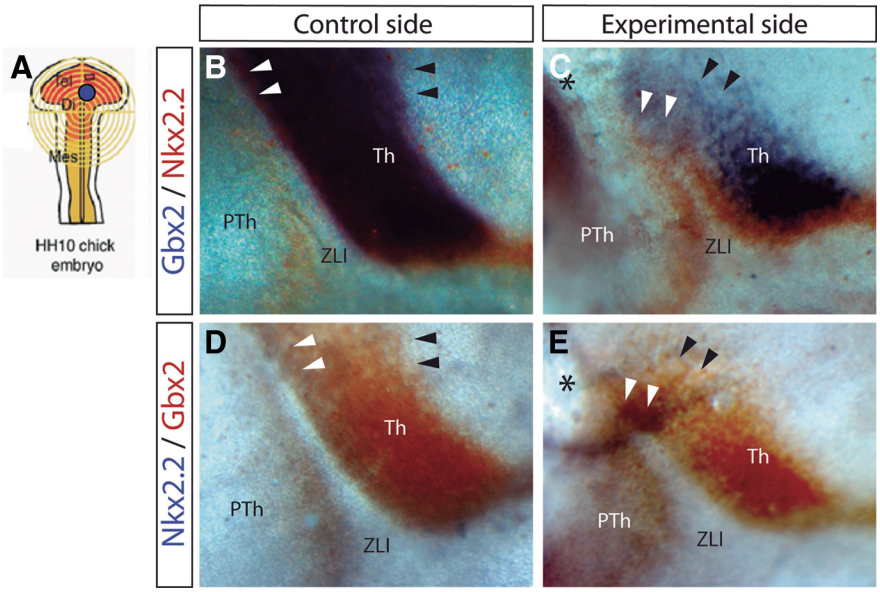

Figure 9. Inhibition of Wnt signal results in downregulation and ectopic expression of *Gbx2* and *Nkx2.2*. ***A***, Schematic representation of the neural tube explants in which Dkk-1 was inserted. ***B–E***, Lateral view of the control side (***B***, ***D***) and experimental side (***C***, ***E***) of chick embryos neural tube analyzed by ISH for *Gbx2* in blue and *Nkx2.2* in red (***B***, ***C***) and for *Nkx2.2* in blue and *Gbx2* in red (***D***, ***E***). Black arrowheads localize the caudal limit of *Gbx2* expression (see its reduction in ***C*** and ***E***), whereas white arrowheads localize the region in the dorsal ZLI in which *Gbx2* and *Nkx2.2* were ectopically expressed. Black asterisks indicate the place in which Dkk-1-soaked beads were implanted. Th, Thalamus; PTh, prethalamus.

Molecular mechanisms positioning the ZLI

Analogously to the mechanism regulating the position and fate specification of the isthmic organizer at the interface of *Otx2*- and *Gbx2*-expressing domains (midbrain/hindbrain), the diencephalic ZLI was proposed to result from the interaction between prechordal (*Six3*- and *Fez*-positive) and epichordal (*Irx3*-positive) regions of the forebrain [Kobayashi et al., 2002; Vieira et al., 2005, Guinazu et al., 2007; note that a new definition of the epichordal floor plate, bringing its rostral end to the mammillary midline (Puelles et al., 2012), implies that the so-called "prechordal territory" now represents the rostral forebrain]. Nevertheless, *Six3* is not required for the ZLI formation in mice (Lagutin et al., 2003; Lavado et al., 2008). Thus, ZLI develops at the interface between prethalamic and thalamic prosencephalic regions expressing heterogeneous TFs that may contribute to ZLI dimensioning in width and confer differential competence to respond to any organizer signals (Vieira and Martinez, 2006).

In the chick neural tube, *Wnt8b* expression represents the earliest distinct marker for the diencephalon. Between HH10 and HH15, its transverse wedge-shaped expression domain collapses into a narrow strip along the ZLI, which contracts from ventral to dorsal. This collapse may be the result of repressive effects occurring within the flanking domains, after activation of TFs such as *Fez*, *Lrrn1*, *Otx2*, and *Irx3*, or attributable to mutual inhibition with *Gli3* (the latter is repressed at the center of the *Wnt8b* domain, whereas *Wnt8b* is downregulated at the periphery). Later on, between HH17 and HH25, the ZLI begins to express *Shh* (Fig. 3). Therefore, the early and dynamic expression of *Wnt8b* in the diencephalic wall may represent the initial condition for p3/p2 boundary positioning and ZLI formation.

Kiecker and Lumsden (2004) proposed that the dynamic expression pattern of *L-fng* in the diencephalon (with progressive encroachment on the forming ZLI) plays a key role in the induction of *Shh* in the ZLI. Because *L-fng* encroachment is not attributable to either cellular movements in the epithelium (Garcia-Lopez et al., 2004) or cell death (Zeltser et al., 2001), it must be the consequence of progressive rostralward induction of *L-fng* into thalamic areas, starting from an initial pretectal (p1) diencephalic alar territory (Zeltser et al., 2001; Ferran et al., 2007). *L-fng* expression in the prethalamus does not change, so that all the encroachment occurs caudal to the ZLI. *L-fng* probably expands into the thalamus region in the wake of the collapsing *Wnt8b* domain at the ZLI, because *Wnt8b* is needed for *L-fng* expression, as we showed. This interpretation suggests that *L-fng* plays a passive role relative to the formation of the ZLI, even if it later modulates its *Shh* expression and boundary properties. Although the contracting *L-fng*-negative neuroepithelium might be seen as a domain permissive for *Shh* expression, this is contradicted by the lack of early induction of *Shh* within it, probably because of the widespread alar expression of *Gli3*, a result of early ventrodorsal patterning. Secondary repression of *Gli3* along the center of the transverse *Wnt8b* expression domain, first visible at HH15, seems instead crucial to allow the *Shh*-positive basal domain to selectively induce *Shh* homeotically within the *Gli3*-devoid stripe.

Interestingly, whereas in the chicken ZLI planar induction out of the *Shh*-expressing basal domain is required for the dorsalward progression of *Shh* expression (Vieira and Martinez 2006; present results), a dorsal Wnt signal (probably accompanied by *Gli3* repression) seems sufficient to activate *Shh* at the ZLI locus in the *one-eye-pinhead* zebrafish mutant, which lacks a basal plate (Scholpp et al., 2006). Species differences in the function of a specific enhancer regulating *Shh* expression at the ZLI (Jeong et al., 2011) might be the cause of this discrepancy.

Other signals contribute to define the width of the ZLI compartment

In this work, we propose that the ZLI compartment expressing *Shh* is positioned and crucially allowed to emerge by Wnt-mediated signals leading to localized repression of *Gli3* rather than by dynamic expression of *L-fng* (Zeltser et al., 2001). An additional role may be played by the early expression of *Lrrn1* just rostral to the ZLI; the latter precedes *Shh* induction and intrigu-

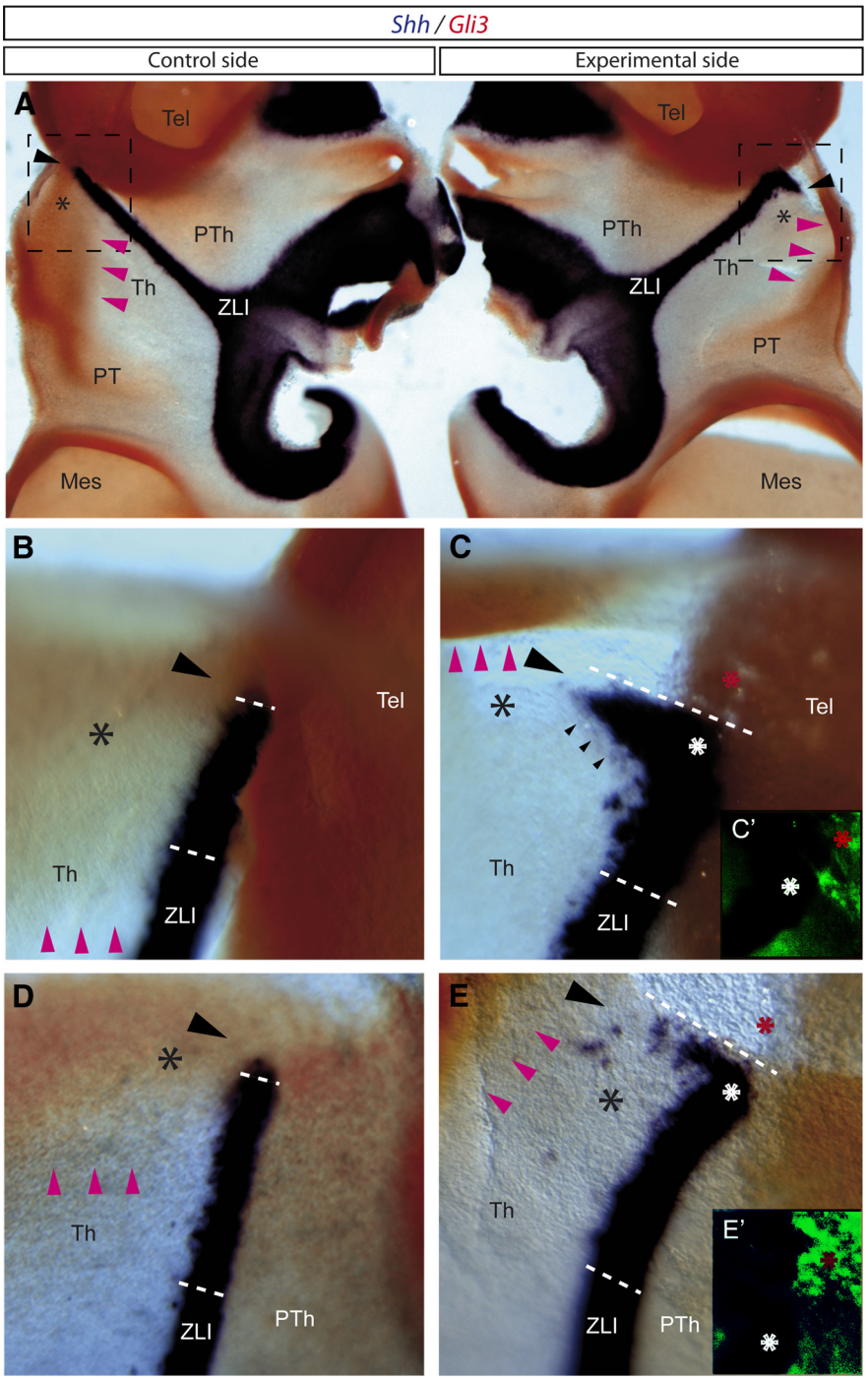

Figure 10. Transplantation of Wnt1-expressing cells results in repression of *Gli3* expression and induction of *Shh* expression in the dorsal region of the thalamus. ***A–E***, Embryos analyzed by ISH for *Shh* in blue and *Gli3* in red. ***A***, Dorsal view of embryos (opened through the midline) analyzed by ISH for *Shh* and *Gli3*. ***B***, ***C***, Magnification of the squares represented in ***A***. ***B***, ***D***, Control side of embryos transplanted with Wnt1-expressing cells. ***C***, ***E***, Experimental side of embryos transplanted with Wnt1-expressing cells. ***C′***, ***E′***, Immunofluorescence for QCPN. Black asterisks mark the most dorsal region of the thalamus, in which *Gli3* is normally expressed (see control side in ***A***, ***B***, ***D***). Pink arrowheads label the ventral limit of *Gli3* expression in the thalamus. Increasing negative gap of Gli3 expression is observed in the experimental side of the embryos (see pink arrowheads in ***C*** and ***E*** compared with ***B*** and ***D***, respectively). Black arrowheads indicate the region in which ectopic *Shh* expression is observed near to the place where Wnt1-expressing cells were transplanted (red asterisks in ***C***, ***C′***, ***E***, ***E′***). White asterisks (***C***, ***E***) indicate the most dorsal domain of the ZLI. Mes, Mesencephalon; Tel, telencephalon; Th, thalamus; PT, pretectum; PTh, prethalamus.

ingly is found likewise at the isthmic organizer (García-Calero et al., 2006; Andreae et al., 2007; Tossell et al., 2011).

Shh is not expressed throughout the ZLI in the *LRP6* mutant mice (Zhou et al., 2004; LRP6 is a Wnt coreceptor) or after blockage of Wnt signaling (present results; Fig. 5*F–I*), and normal Wnt signaling at early neural tube stages seems to be required for the establishment of an *Shh/Gli3*-negative primordial ZLI region at the alar p3/p2 boundary. Guinazu et al. (2007) performed electroporation experiments at stage HH16 using constructs that activated or blocked the Wnt/β-catenin pathway. They reported that thalamic gene expression is not directly or indirectly regulated by Wnt proteins. This result is not contradictory with our conclusion above, because the ZLI probably was already established before Wnt signaling was modified in their experiments. Inhibition of the Wnt pathway once *Shh* is already expressed in the ZLI does not have any effect on its maintenance (Fig. 6).

Other molecular determinants seem to collaborate with *Wnt8b* in the ZLI specification process. It was demonstrated in zebrafish embryos that the prethalamus plays a role in ZLI formation by means of frizzled-receptor-mediated blockage of Wnt signaling (Peng and Westerfield, 2006; Tendeng and Houart, 2006; Jeong et al., 2007). This mechanism would contribute to the shrinkage of *Wnt8b* expression, thus dimensioning the width of the ZLI, together with caudal signals such as *Irx3* acting at the thalamic side (p2). The latter effect was evidenced in our grafts of prospective ZLI tissue into caudal diencephalon, which lost their *Wnt8b* expression. Moreover, in zebrafish, *Otx1l* and *Otx2* expression in the diencephalon determines a competent domain in which a ZLI is induced (Scholpp et al., 2007). However, the homogeneous expression of *Otx2* throughout the chick diencephalon at stages HH15–HH17 does not suggest a relevant positional role of this gene in specifying the neuroepithelial corridor in which *Shh* will be induced, although it may be needed to underpin the interactions between *Wnt8b* and the other protagonists, including *Gli3*. As the ZLI matures, after *Shh* expression begins, *Otx2* expression is upregulated at its neuroepithelium (Crossley et al., 2001).

Wnt/β-catenin, L-fng, and Shh/Gli signaling pathway during the establishment of *Shh* expression in the ZLI

Previous studies have demonstrated that *Gli3* is required for *Wnt* gene expression at various brain locations (Grove et al., 1998; Mullor et al., 2001; Ulloa et al., 2007). Moreover, Alvarez-Medina et al. (2008) showed that Wnt activity is required for *Gli3* expression. In contrast to this positive mutual regulation, at the chicken ZLI, the *Gli3* and *Wnt8b* genes are complementarily expressed.

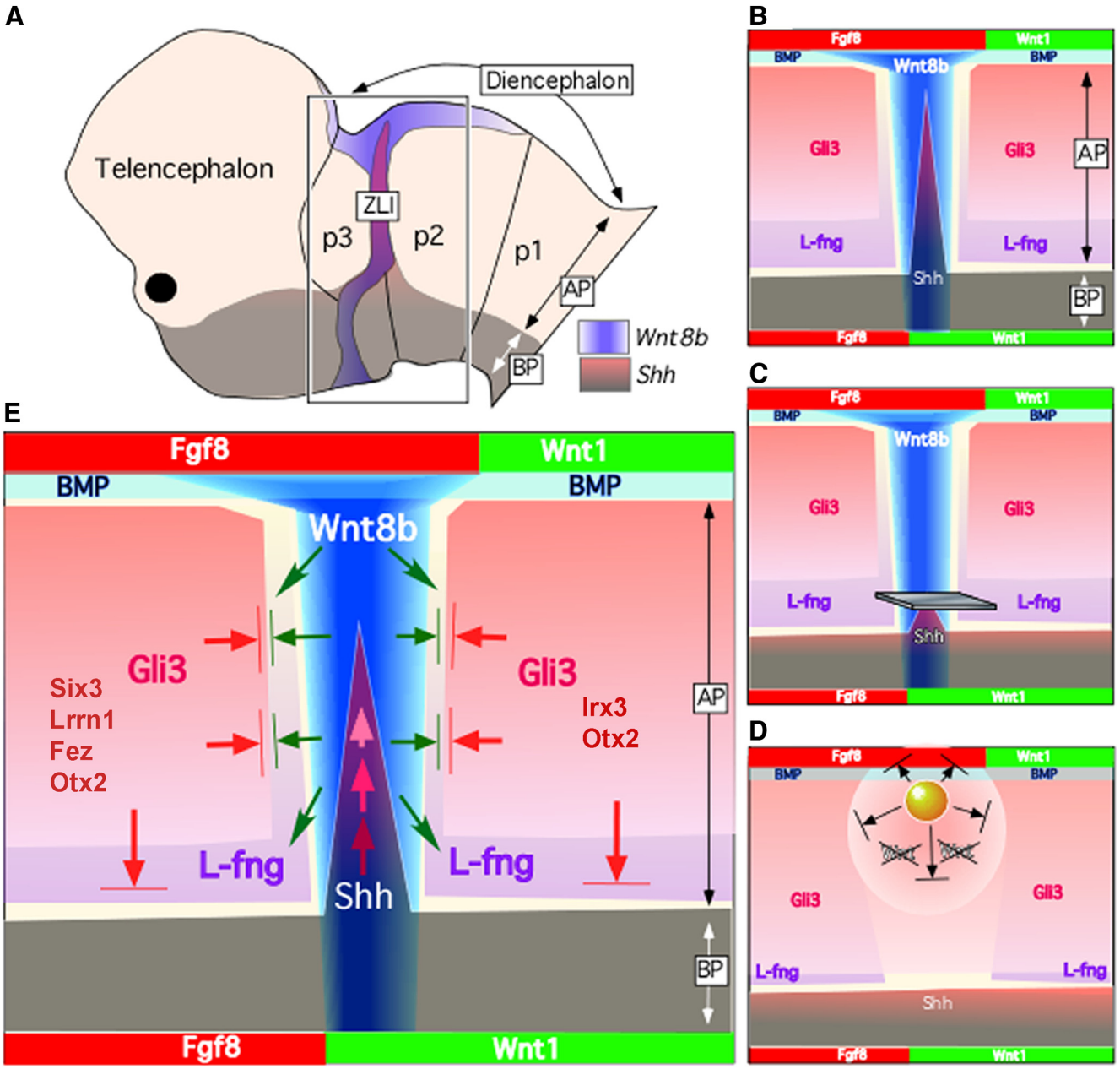

Figure 11. Schematic representation illustrating regulatory interactions between morphogenes and TFs acting in the ZLI. ***A***, Drawing of chick embryo prosencephalon (stage HH23) showing the relationship of the ZLI with diencephalic prosomeres. *Wnt8b* and *Shh* expression have been represented by color codes. ***B***, Gene expression pattern of different genes (names and color codes) in the diencephalic neuroepithelium. ***C***, Gene expression pattern in the diencephalon after *Shh* abolition in the ZLI by metal microbarrier insertion, showing that the limit between p2/p3 was not disrupted: *Wnt8b* was normally expressed in the ZLI, and *L-fng* showed normal expression patterns. ***D***, Gene expression pattern in the diencephalon after *Wnt8b* inhibition in the dorsal ZLI by Dkk-1-soaked bead implantation. The negative gap of *Gli3* expression in the ZLI was abolished. In the contrast, encroachment of *L-fng* expression was impeded. These two effects, mediated by Wnt8b signal, impede *Shh* progression in the ZLI from the basal plate. ***E***, Mechanicistic model interpreting our results: Wnt8b morphogenetic gradient from the ZLI (blue gradient) controls the expression of diencephalic genes and is fundamental for the correct specification of the *Shh* expression in the ZLI in chick embryos. The arrows show interactions (activation or repression) between diencephalic signals: green arrows represent *L-fng* induction, and green arrows with lines represent *Gli3* inhibition by Wnt8b signaling. Then, the negative gap of *Gli3* expression and an encroachment of *L-fng* expression in the ZLI generated a permissive territory (free from repressive activity of Gli3, red arrows with lines) for *Shh* expression in this region, which progresses by planar auto-inductive mechanisms from the basal plate (red arrows). ap, Alar plate; BP, basal plate.

When Wnt canonical signaling does not oppose the expression of *Gli3* at the prospective ZLI, *Shh* is not activated in the alar diencephalon (Fig. 5). Thus, because the expression of *Gli3* (the main transcriptional repressor of the Shh/Gli pathway) in the ZLI inhibits *Shh* induction, the observed early downregulation of *Gli3* by Wnt8b signal is a key process in the diencephalon for establishment of the ZLI organizer and secondary induction of *Shh*. Although Wnt8b signal seems necessary for positionally restricted downregulation of *Gli3* at the primordial ZLI, it is not sufficient, as is indicated by the fact that the whole domain that expresses *Wnt8b* does not lose *Gli3* expression. A combination of factors acting at the early interface between prethalamic (*Six3*$^+$, *Lrrn1*$^+$, *Fez*$^+$) and thalamic (*Irx3*$^+$) prosencephalic neuroepithelium apparently contributes to the dimensioning of the *Gli3*-negative primordial ZLI.

We conclude that *Wnt8b* expression in the alar diencephalon underlies jointly with other molecular interfaces (*Fez*, *Lrrn1*, *Otx2*, and *Irx3*) the p3/p2 boundary formation and subsequently

underpins at its center the required molecular events that result in a permissive *Gli3*-negative transverse spike (the primordial ZLI), in which basal plate inductive signals (Shh) generate the definitive ZLI (Fig. 11). The results described here demonstrate a regulation of *L-fng* and *Gli3* expressions by Wnt signaling in the alar diencephalon. *L-fng* modulates Notch signaling at the boundary cells (Tossell et al., 2011), thus contributing to specification of the ZLI borders (ZLI width; Larsen et al., 2001; Zeltser et al., 2001). In parallel, by repressing *Gli3* expression, Wnt signaling crucially generates a permissive territory at the p3/p2 boundary, the primordial ZLI, in which *Shh* results activated ventrodorsally by planar inductive mechanisms (Fig. 11).

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