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Expression dynamics of the LIM-homeobox genes, *Lhx1* and *Lhx9*, in the diencephalon during chick development

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ABSTRACT The diencephalon is the caudal part of the developing forebrain, which corresponds to prosomeres 1 to 3. The mature diencephalon is functionally and anatomically divided into well-defined nuclei. Previous researches have shown that LIM-homeobox genes are important transcription factors during diencephalon regionalization in mice. Here we examined expression patterns of several chick orthologs of LIM-homeobox genes. *Lhx1* and *Lhx9* were expressed in the diencephalon from early stages and their expression in the diencephalon became restricted to prosomeres 1 and 2 in distinct fashions. Then we also studied the regulatory effects of possible upstream signals by *in ovo* electroporation. *Lhx1* was found to be up-regulated by Shh signaling. Whereas *Lhx9* was up-regulated by *Wnt3a* and *Fgf15*, it was down-regulated by *Shh*. Our data suggest that the LIM-homeobox genes, *Lhx1* and *Lhx9*, regulated by ventral and/or dorsal signals, may play important roles in controlling regionalization of the diencephalon during chick development.

KEY WORDS: *lhx1, lhx9, chick, diencephalon regionalization, in ovo electroporation*

Introduction

In vertebrates, the anterior neural epithelium undergoes morphological subdivisions to generate vesicle-like structures known as the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). In chick, the prosencephalon has become further divided into the telencephalon and diencephalon by HH stages 12-13. The diencephalon is the caudal part of the forebrain. Its primordium consists of three neuromeric structures, called prosomeres (P; Puelles and Rubenstein, 1993). The mature diencephalon is functionally and anatomically divided into well-defined nuclei. The LIM-homeodomain family of transcription factors, Lhx1/5 and Lhx2/9, as well as Gbx2, Pax6 and Neurogenin2 (Ngn2), have been thought to play important roles in diencephalon regionalization in mice (Fujii *et al.,* 1994; Sheng *et al.,* 1997; Retaux *et al.,* 1999; Bulfone *et al.,* 1993; Miyashita-Lin *et al.,* 1999; Walther and Gruss, 1991; Stoykova and Gruss, 1994; Stoykova *et al.,* 1996; Kawano *et al.,* 1999; Gradwohl *et al.,* 1996; Sommer *et al.,* 1996; Nakagawa and O'Leary, 2001). These regulatory genes are expressed in distinct yet often overlapping patterns, and may cooperate to control specification and differentiation of the thalamic nuclei and cell types (Nakagawa and O'Leary, 2001). We thus presumed that their orthologs play similar roles in development of the chick diencephalon.

Lhx1/*Lim1* was firstly studied in the Spemann organizer in *Xenopus* (Taira *et al.,* 1992), and then in the brain and node in mice (Fujii *et al.,* 1994). In chick, it was expressed in motor neurons and interneurons throughout the spinal cord (Tsuchida *et al.,* 1994). It has also been known as a good marker for the pretectum anlagen or P1 (Matsunaga *et al.,* 2000). To date, however, the spatial and temporal expression patterns of *Lhx1* have not been analyzed in detail in the chick diencephalon. Chick *Lhx9* (also known as *cLhx2b*) was firstly reported to be detectable in the anterior limb bud, and was shown to play an important role in determination and specification of the anterior-posterior positional value (Nohno *et al.,* 1997). They also showed the expression patterns of *Lhx9* in the dorsal midbrain,

Abbreviations used in this paper: lhx, lim homeobox; LIM, lin-11/islet-1/mec-3; ngn, neurogenin; shh, sonic hedgehog gene.

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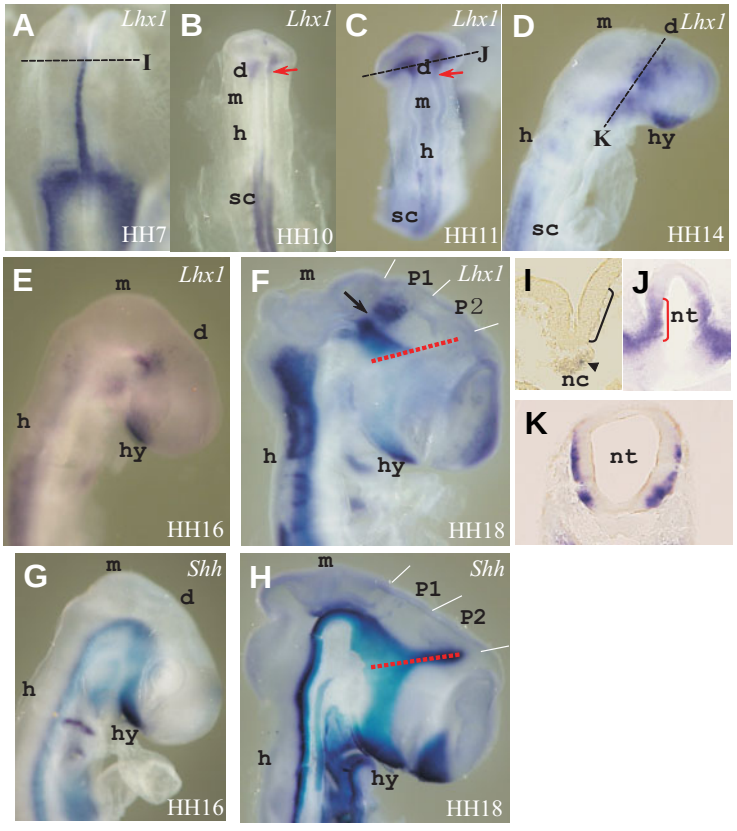
forebrain, and dorsolateral region of the spinal cord and hindbrain. Recently, it was reported that chick *Lhx9* was expressed predominantly in the dorsal diencephalon with the distinct ventral limit in a similar pattern to *Gbx2* (Lim *et al.,* 2002). We examined the expression patterns of several chick LIM-homeobox genes including *Lhx1*, *Lhx2/9*, *Lhx6* and *Lmx1b*. We found that *Lhx1* and *Lhx9* were detected in the developing diencephalon. Then we further studied the regulatory effects by the possible upstream signals, including Sonic hedgehog (Shh), Wnt and Fibroblast growth factor (Fgf). We found that *Lhx*1 was up-regulated by *Shh*, and *Lhx9* was up-regulated by *Wnt3a* and *Fgf15* while down-regulated by *Shh*. These results suggest that LIM-homeobox genes, *Lhx1* and *Lhx9*, detected in the diencephalon, are regulated by the ventral and/or dorsal signals, and that they may play important roles in controlling the diencephalon regionalization during chick development.

Results

Expression patterns of chick Lhx1 *and* Lhx9

Lhx1/*Lim1* was firstly studied in the Spemann organizer in *Xenopus* (Taira *et al.,* 1992), and then in the brain and node in mice (Fujii *et al.,* 1994). In chick, it was expressed in motor neurons and interneurons throughout the spinal cord (Tsuchida *et al.,* 1994). Recently, it was reported that the *Lhx1* expression was detected in the hindbrain from Hamburger and Hamilton (HH) stage 10 onwards (Cepeda-Nieto *et al.,* 2005). Here we studied the expression patterns of *Lhx1* from early stages by whole-mount



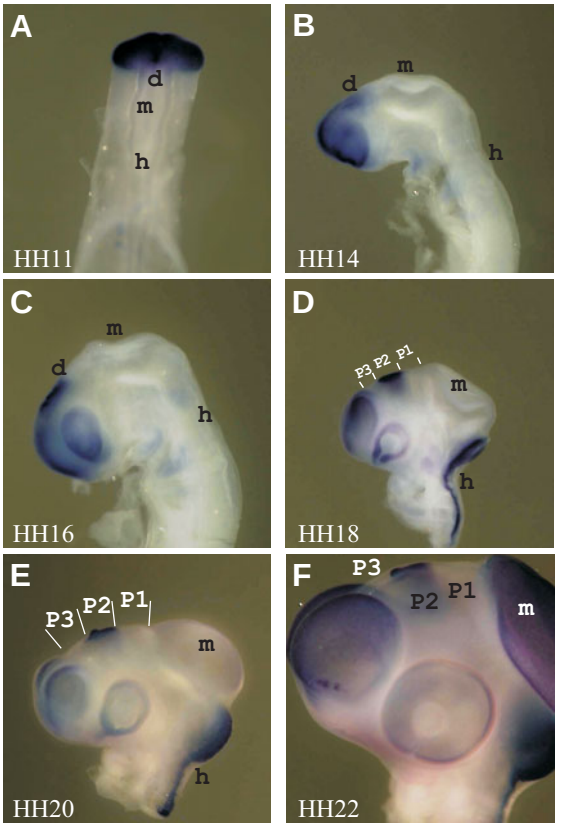


Fig. 1 (Left). Spatial and temporal expression patterns of chick *Lhx1* and comparison with *Shh*. (A-C) *Dorsal and* **(D,E,G)** *lateral views of the embryos stained by whole-mount RNA* in situ *hybridization using the chick* Lhx1 *antisense probe.* **(F,H)** *The embryos had been hemisectioned and viewed from the ventricular side. Sections of whole-mount processed embryos are shown in* **(I-K)**. *At HH stage 7,* Lhx1 *was expressed in the notochord* **(A, I)**. *A black bracket in (I) indicates the neural ectoderm free of* Lhx1 *expression. An arrowhead points to* Lhx1 *expression in the notochord (nc). By HH stage 10, the prospective caudal diencephalon was labeled* **(B)**, *and expression was restricted to the ventral region, as indicated by a red bracket in* **(J)**. *The posterior border of expression coincides with the diencephalon/mesencephalon boundary; see red arrow in* **(B,C)**. *From HH stage14, the expression in P1 extended dorsally and showed a ventral* ^{High}*-dorsal* ^{Low} *gradient* **(D,E,K)**. *At HH stage 18, expression at P1 was clearly divided into ventral and dorsal regions with a gap between them; see black arrow in* **(F)**. *It was also clearly detected in the telencephalic vesicles at this stage (F). The expression patterns of chick* Shh *at HH stage 16 and 18 overlapped with those of* Lhx1 *in the ventral part of P1, hypothalamus and mesencephalon (compare E, F and G, H). Abbreviations: d, diencephalon; h, hindbrain; hy, hypothalmus; m, mesencephalon; nc, notochord; nt, neural tube; P1-3, prosomere1-3; sc, spinal cord; red dashed line in (F,H), zona limitans intrathalamica.*

Fig. 2 (Right). Expression pattern of chick *Lhx9* at HH stages 11-22. *The embryos were stained by whole-mount RNA* in situ *hybridization using the chick* Lhx9 *antisense probe.* **(A)** *The dorsal view shows expression of* Lhx9 *throughout the prosencephalon at HH stage11, being particularly strong in the most anterior region, optic vesicles and prospective dorsal thalamus.* **(B-F)** *Lateral views of the embryos at the indicated stages. Expression in the diencephalon became restricted to the dorsal P2, while weak expression in the dorsal P3 became down-regulated. Abbreviations are as in Fig.1.*

and section *in situ* hybridization. During gastrulation, *Lhx1* was detected in the Hensen's node, notochord and prechordal plate, but not in the ectoderm (Fig. 1A, I and data not shown). As the neural tube closed and optic vesicles began to evaginate, *Lhx1* was clearly detected in the presumptive caudal diencephalon (Fig. 1B, C), and restricted in the ventral region (Fig. 1J). It was also expressed in the caudal hindbrain and spinal cord. From HH stage14, the expression at the ventral region of P1 showed a ventral ^{High}-dorsal ^{Low} gradient (Fig. 1K). By HH stage18, the expression in P1 extended dorsally and was separated into the basal and alar regions with a gap between them (arrow in Fig. 1F). The ventral strip of the expression extended rostrally into P2 and caudally into the mesencephalon, and the dorsal expression region maintained the ventral^{High}-dorsal^{Low} gradient pattern within P1. It was also expressed in the rostral cerebral vesicles and dorsal midline of P2 (Fig. 1F and data not shown). The expression in the hypothalamus was decreased gradually during development (hy in Fig. 1E, F and data not shown). We also examined the expression pattern of *Shh* at HH stage 16 and 18 (Fig. 1G, H), and compared that of *Lhx1* with it. Here we showed that *Lhx1* and *Shh* were expressed in an overlapping pattern in the ventral part of P1, hypothalamus and mesencephalon (Fig. 1E-H).

Previous studies indicated that chick *Lhx9* was expressed in the dorsal midbrain, forebrain, and dorsolateral region of the spinal cord and hindbrain (Nohno *et al.,* 1997). It was also expressed predominantly in the dorsal diencephalon with the distinct ventral limit of expression (Lim *et al.,* 2002). Here we found that *Lhx9* was expressed throughout the prosencephalon and optic vesicles at HH stage11 (Fig. 2A). The expression became gradually restricted to the rostro-dorsal telencephalon, dorsal P2 and ventral side of the eye, while the weak expression in the dorsal P3 was down-regulated and disappeared by HH stage18 (Fig. 2B-F). It was also detectable in the dorsolateral region of the hindbrain and spinal cord from HH stage18 onwards (Fig. 2D-F), which was consistent with the observations reported by Nohno *et al.* in 1997. Taken together, the expression patterns of the two LIM-homeobox family members suggest the existence of regulation along the rostral-caudal and dorsal-ventral axes of the diencephalon. This promoted us to investigate the regulatory effects of potential upstream signals on regionalization of diencephalon.

Up-regulation of Lhx1 *and down-regulation of* Lhx9 *by* Shh *overexpression*

In the spinal cord, inductive signals from the roof plate and floor plate control neuronal fates along the dorsoventral axis (Tanabe and Jessel, 1996; Lee and Jessel, 1999). Signals from the roof plate, such as TGFβ family members, are required in the dorsal spinal cord for the induction of *Lhx2* and *Lhx9* (Liem *et al.,* 1997; Lee and Jessel, 1999; Lee *et al.,* 2000). In the ventral spinal cord, distinct classes of motor neurons and ventral interneurons are generated by a graded signaling activity of Shh (Briscoe *et al.,* 1999, 2000). Previous studies indicated that Pax6 and Nkx2.2 appeared to be essential intermediaries for Shh to regulate the differential expression of LIM-HD proteins, including Lhx1, Lhx3, Lhx4, Lhx5, Isl1 and Isl2 (Ericson *et al.,* 1997; Briscoe *et al.,* 1999). It was also implicated that regionalization of the diencephalon might be established by mechanisms similar to those in the spinal cord (Nakagawa and O'Leary, 2001). Thus, we investigated the regulatory effects by the dorsal and ventral signals on chick *Lhx1* and *Lhx9*.

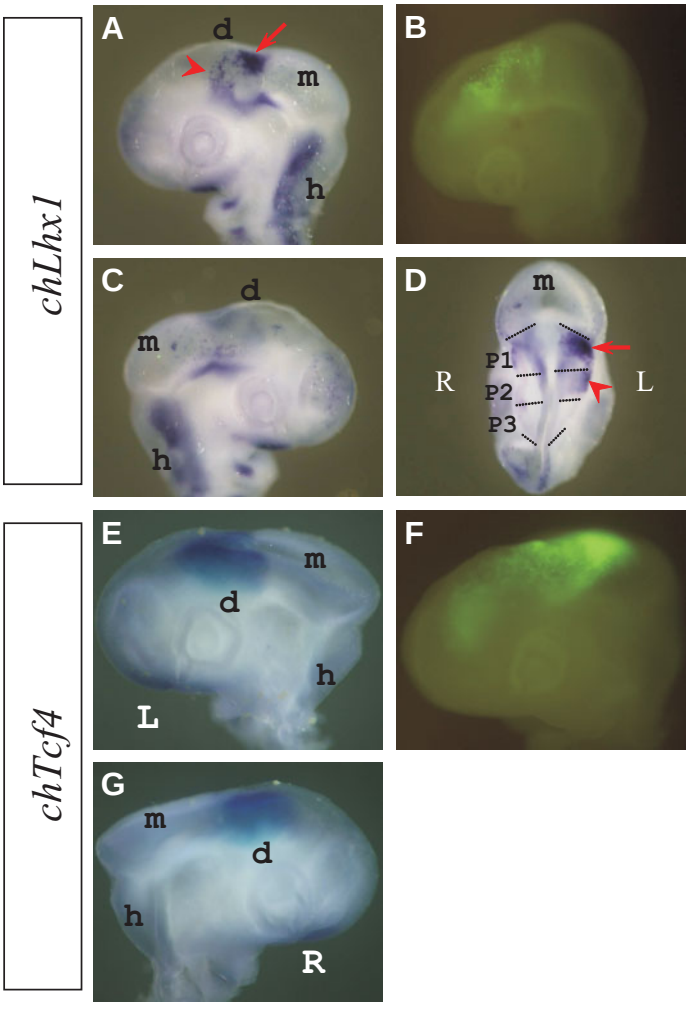


Fig. 3. Induction of *Lhx1* and effect on *Tcf4* of *Shh* overexpression. *pCIG-Shh was electroporated at HH stage 10-11.* In situ *hybridization was performed at HH stage 18 with the probes indicated in the left columns.* **(A,B)** *The region of* Lhx1 *ectopic induction is consistent with that of GFP expression. The level of* Lhx1 *expression is elevated in the dorsal part of P1, see arrow in* **(A,D)**. *Ectopic expression of* Lhx1 *is induced in the lateral part of P2; see arrowhead in (A,D).* **(E-G)** *Overexpression of* Shh *did not alter* Tcf4 *expression in the P1 and P2 alar plates. Note that* Tcf4 *expression on the electroporated side (E, left indicated by "L") was comparable to that on the control side (G, right indicated by "R"). Abbreviations are as in Fig.1.*

Ectopic expression of *Shh* increased *Lhx1* expression strongly and induced ectopic expression in the dorsal P1, still, leaving the gap between the ventral and dorsal expression regions at 30 hours after electroporation (Fig. 3A, C, D; n=8/8). It also induced ectopic expression in the dorsal mesencephalon (data not shown; n=1). However, it remained unknown whether this up-regulation of *Lhx1* in the dorsal P1 was a secondary effect of ventralization of the alar plate by *Shh*. So we examined the expression of *Tcf4*, the alar plate marker of P1 and P2, after electroporation of the *Shh* expression vector into the dorsal diencephalon. Overexpression of *Shh* did not alter the *Tcf4* expression domain at all (Fig. 3E, G;

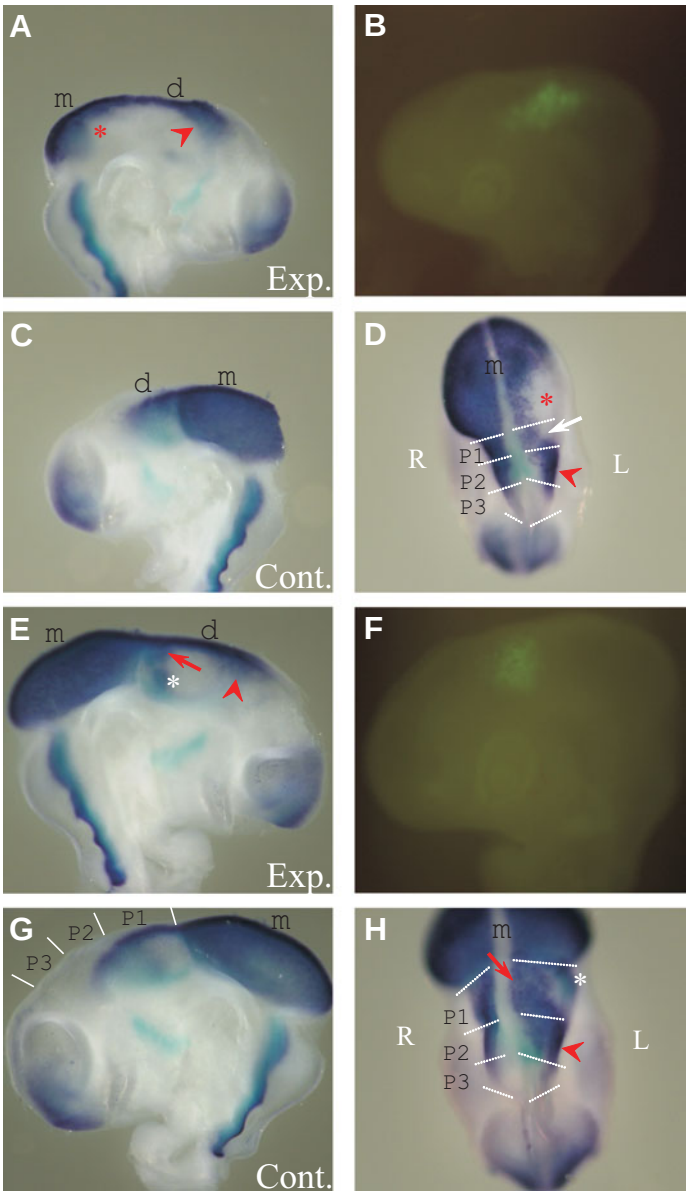


Fig. 4. Regulation of chick *Dbx1* by *Shh* overexpression. *pCIG-Shh expression vector was electroporated at HH stage 10-11.* In situ *hybridization was performed at HH stage 18* **(A-D)** *and HH stage 19* **(E-H)**. **(A,C,E,G)** *The embryos had been hemisectioned and viewed from the ventricular side. Expression of* Dbx1 *was down-regulated in the anterior mesencephalon; see red asterisk in* **(A,D)**, *and P1; see white arrow in* **(D)** *on the electroporated side (indicated as Exp.) compared to the control side (Cont.). The red arrow in* **(E,H)** *points to laterally extended expression and the white asterisk indicates ectopically induced expression in the P1 alar plate. The red arrowhead in* **(A,D,E,H)** *indicates up-regulated expression in the dorsal P2. Note that* Dbx1 *was not expressed in the roof plate. Abbreviations are as in Fig.1.*

n=6/6). This result suggests that the alar plate was not ventralized after *Shh* overexpression and supports the idea that the domain of induced or up-regulated *Lhx1* expression in the dorsal P1 by *Shh* retains the dorsal character in terms of *Tcf4* expression. We also analyzed the expression of chick *Dbx1*. It was strongly expressed in the dorso-lateral wall of the mesencephalon and extended in a strip into P2, parallel to the dorsal midline. In the diencephalon, it was detected in the zona limitans intrathalamica (ZLI), the alar zone starting near the rostral boundary of the dorsal thalamus and epithalamus and extending through the middle of the pretectum. In the caudal half of the pretectum, *Dbx1* expression was confined to the boundary between alar and basal plates, in addition to the dorsal strip (Fig. 4C, G). We examined four embryos and found that all of them showed down-regulated expression in the mesencephalon and the rostral P1 (Fig. 4A, D and data not shown). On the other hand, in one of them, the expression in the dorsal region of the caudal P1 was extended laterally (Fig. 4E, H, red arrow), and an ectopically induced expression was observed in the dorso-lateral region of the caudal P1 (Fig. 4E, H, white asterisk). In all of the four embryos, the expression in the alar plate of P2 was up-regulated (Fig. 4A, D, E, H and data not shown). So, considering the above data, it is yet difficult to give a conclusion whether ectopically-*Lhx1* expressing cells in P1 are “ventralized”.

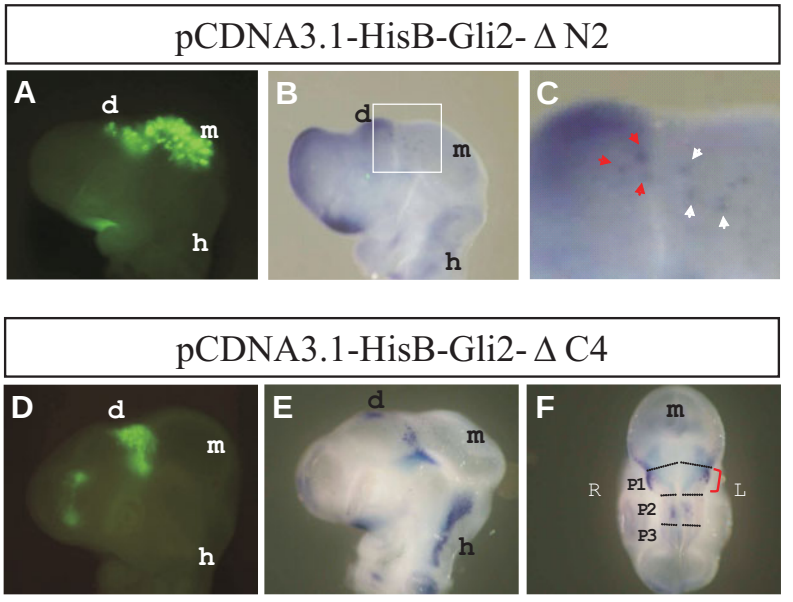
It has been demonstrated that Gli2 is composed of positive and negative regulatory domains. In the absence of Shh, Gli2 is thought to be truncated to become a repressor form, while full-length Gli2 acts as an activator in the presence of Shh. Removal of the repression domain at the N-terminus converted Gli2 into a constitutive activator (Gli2-ΔN2, Sasaki *et al.,* 1999). In transgenic mouse embryos, Gli2–ΔN2 could mimic the effect of a Shh signal, implying a key mechanism of Shh signaling through modulation of the N-terminal repression domain of Gli2 (Sasaki *et al.,* 1999). Here we showed that the Gli2–ΔN2 induced ectopic *Lhx1* expression in the P1 and mesencephalon (Fig. 5A, B, C), which is consistent with the observations in transgenic mice by Dr. Sasaki research group. On the contrary, *Lhx1* expression in P1 on the experimental side was decreased at 30 hours after electroporation of the repressor form of Gli2 (Gli2–ΔC4: C-terminal truncated Gli2) into the caudal diencephalon (Fig. 5D, E, F; n=3/5). Although Gli1 is thought to function only as an activator in the Shh signaling pathway, our data indicated that no obvious change in *Lhx1* expression was detected after overexpression of*Gli1* (data not shown).

In contrast, the expression of *Lhx9* in the dorsal P2 disappeared after overexpression of *Shh*, which indicates the repressive effect of Shh on *Lhx9* (Fig. 6A, C, D; n=2/3).

We also examined the effect of Wnt1, Wnt3a or Fgf15 on the expression of *Lhx1* (n=3, each), but we found that there was no obvious change.

Up-regulation of Lhx9 *expression by Wnt3a and Fgf15 signals*

Secreted signaling factors of Wnt family are expressed in the posterior diencephalon before HH stage18 and have been implicated in diencephalic regionalization (Braun *et al.,* 2003; Garcia-Lopez *et al.,* 2004). Here, we examined the effects of two members of Wnt family, Wnt1 and Wnt3a. *Wnt1* was first expressed in the region of the presumptive mesencephalon of the head-fold, while *Wnt3a* was first observed in the rhombencephalic regions of the open neural fold. After neural tube closure, both *Wnt1* and *Wnt3a* were expressed in partially overlapping domains in the mesencephalon and caudal diencephalon, and then became restricted to the dorsal midline (Hollyday *et al.,* 1995). Therefore, Wnt1 and 3a are thought to be dorsal signals.



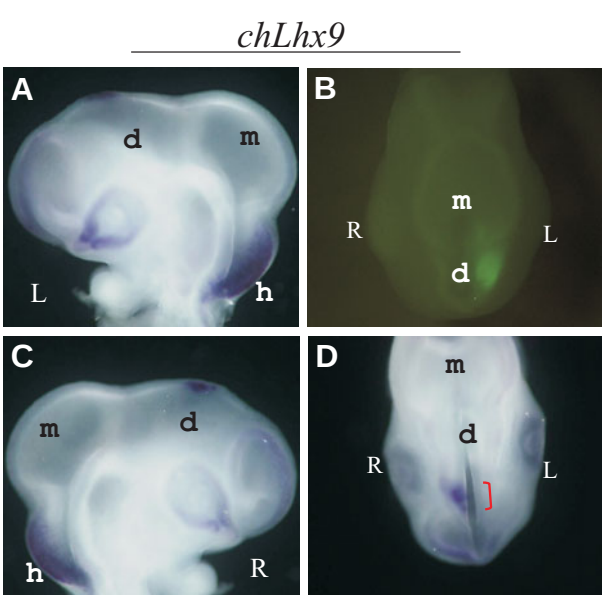


Fig. 5 (Left). Effects of activator and repressor forms of Gli2 on *Lhx1* expression. *The embryos were fixed at 30 hours after electroporation.* **(A,D)** *GFP expression on the electroporated side (left).* **(A-C)** *The activator form of Gli2 (Gli2–ΔN2) induced ectopic* Lhx1 *expression in small patches in P1 (red arrowheads in C), and mesencephalon (white arrowheads in C), indicating that it could mimic the effect of* Shh. **(C)** *High power magnification of the boxed area in (B).* **(E,F)** *The repressor form of Gli2 (Gli2–ΔC4) caused an opposite result, decreasing the expression of* Lhx1. *A red bracket on the electroporated side (L, left) indicates the expression range counterpart in P1 of the control side (R, right) to illustrate decreased expression. Note that anterior expression in the bracket was abolished on the left side. Abbreviations are as in Fig.1.*

Fig. 6 (Right). Repression of *Lhx9* by *Shh* overexpression, *using pCIG-Shh vector.* In situ *hybridization at HH stage 18. In contrast to* Lhx1, Lhx9 *expression in the dorsal P2 disappeared after overexpression of* Shh **(A,D).** *A red bracket in (D) indicates that the original expression area in P2 disappeared on the experimental side.* **(B)** *GFP illustrated the domain of ectopic* Shh *expression.* **(C)** Lhx9 *expression of the control side. Abbreviations are as in Fig.1.*

Overexpression of *Wnt3a* increased the expression level of *Lhx9* in the dorsal P2 (asterisk in Fig. 7A, D; n=4/6). In addition, the expression domain was extended rostrally into P3 (arrow in Fig. 7D) and caudally into P1 (Fig. 7D and data not shown). On the other hand, overexpression of *Wnt1* did not show any effect on *Lhx9* expression (data not shown).

TCF4 is one of the down-stream transcription factors in a canonical Wnt signaling pathway, and its endogenous expression is detected in the dorsal diencephalon. Thus, we examined whether the dominant negative form of *Tcf4* (*dnTcf4*) has an opposite effect on *Lhx9* expression to *Wnt3a*. After overexpression of pCIG-dnTcf4 into the developing diencephalon, the dorsolateral *Lhx9* expression in the dorsal P2 was suppressed as expected (Fig. 7H; n=4/6). Our data suggest that *Wnt3a*, not *Wnt1*, is sufficient to increase *Lhx9* expression through the canonical pathway in the dorsal P2.

In the chick, *Fgf19* expression was detectable in the dorsal diencephalon from HH stage14 (Kurose *et al.,* 2004). This suggests a possible regulatory relationship between *Fgf19* and the expression of *Lhx9*. It has been known that *Fgf15* is the rodent ortholog of *Fgf19* of chick because of their syntenic location in the genome (Katoh and Katoh, 2003). Moreover, *Fgf15* mimics *Fgf19* activity to induce expression of otic markers in a chick explant assay, suggesting that they have similar physiological activities (Wright *et al.,* 2004). We therefore used the mouse *Fgf15* expression vector to examine positive regulatory effects on *Lhx9* expression. After *in ovo* electroporation of the *Fgf15* expression vector, increased expression of *Lhx9* was observed in the slightly expanded domain (compare Fig. 8A and C; D; n=3/3). Our data provide further evidence that the physiological activities are conserved between mouse FGF15 and chick FGF19. The results also confirm the up-regulation effect on *Lhx9* expression. Thus, signals from the roof plate, such as WNT and FGF are important in the dorsal diencephalon for regulation of *Lhx9*. Because Wnt3a and Fgf15 both up-regulated the expression of *Lhx9,* which promoted us to examine whether there is synergism between them. However, we did not find obvious up-regulation effect on *Lhx9* expression after coexpression of Wnt3a and Fgf15 (data not shown).

Discussion

In this study, we have shown that (1) chick *Lhx1* and *Lhx9*, just like the orthologs in *Xenopus* and mice, can be used as markers for P1 and P2, respectively, (2) *Lhx1* is up-regulated by Shh signaling, (3) *Lhx9* is up-regulated by *Wnt3a* and *Fgf15*, and down-regulated by *Shh*. Here we will discuss the patterning mechanisms in controlling the diencephalon regionalization during chick development.

Lhx1 *is one of the region-specific genes to determine the identity of the pretectum*

On the basis of analysis of morphology, molecular markers, and boundary characteristics, the alar plate of the diencephalon is progressively subdivided to form three distinct regions: the pretectum (P1), dorsal thalamus (P2) and ventral thalamus (P3).

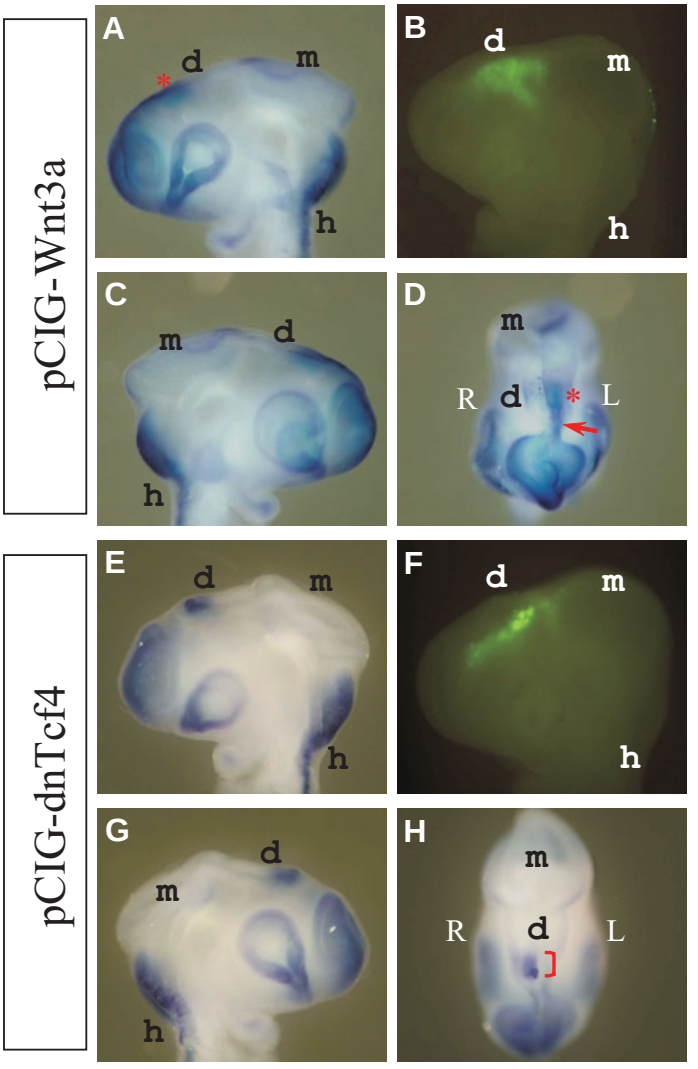


Fig. 7. Positive-regulation of *Lhx9* expression by *Wnt3a*. (A,E) *Lateral views of* Lhx9 *expression on the experimental side.* **(C,G)** *Lateral views of* Lhx9 *expression on the control side. Overexpression of* Wnt3a *increased the level of dorsal expression of* Lhx9 *in P2 (asterisk in A,D). In addition, the expression domain expanded laterally and anteriorly into P3 (arrow in D, dorsal view). On the contrary,* Lhx9 *expression in the dorsal P2 was suppressed after electroporation of pCIG-dnTcf4 (H, red bracket, dorsal view).* **(B,F)** *GFP indicates the domain of electroporation. The difference is not very obvious in the lateral views. Abbreviations are as in Fig.1.*

It has been shown that the first morphological subdivision is observed at HH stage16 when P1 becomes distinct from P2 by adopting neuromeric morphology and expressing *Prox* (Larsen *et al.,* 2001). In the present study, we showed that the expression of *Lhx1* was already detected in the caudal diencephalon by HH stage10. From HH stage14, *Lhx1* was strongly expressed in the basal plate of P1 and weakly expressed in the alar plate. Our data showed that the expression of *Lhx1* preceeds that of *Prox* by several stages, which indicated a possibility that *Lhx1* is one of the upstream genes to decide the identity of P1. Furthermore, after comparing the expression pattern of *Lhx1* with that of *Shh*, it strongly suggests a possibility that *Lhx1* may be induced by a Shh signal. However, from HH stage18, the expression in P1 was divided into ventral and dorsal regions with a gap between them (arrow in Fig. 1D). This result raised two possible explanations. In the first case, both of the ventral and dorsal expressions of *Lhx1* in P1 were induced by Shh from the basal plate, because it showed a ventral^{High}-dorsal^{Low} gradient pattern from early stages (Fig.1B, C). It has been reported that in the midbrain, hindbrain and spinal cord, *Dbx* and *Dbx2*, the two members of the homeobox gene family *Dbx*, are expressed in the boundary separating the basal and alar plates, which seems to correspond to the sulcus limitans (Shoji *et al.,* 1996). In our result, the gap between the two expression domains also seemed to correspond to the sulcus limitans. Expression of chick *Dbx1*, which was somewhat weak, as occurs in mouse, was detected in the region between the basal and alar plate in the caudal diencephalon (Fig. 4 G and data not shown). Our data raise the possibility that *Dbx1* may be involved in the appearance of *Lhx1* expression gap. Therefore, this region seems to have unique characters, which are different from those of the alar and basal plates. The increased expression in the dorsal P1 may result from up-regulation and maintenance by other factors following the initial expression by Shh. In the second case, the ventral and dorsal expressions are induced by Shh from the floor plate and ZLI, respectively. Initially, Shh from the ZLI would induce the expression in the alar plate of P2 and P1, while some unknown genes may inhibit expression in P2, permitting P1 expression. Considering a V-D gradient pattern, not an A-P gradient pattern, we prefer the first possibility to the second one.

Lhx1 *is ectopically induced by* Shh *overexpression in the diencephalon*

Shh is produced in the notochord and floor plate, and is implicated to induce ventral cell types in the CNS (Tanabe and Jessell, 1996). *In vivo* misexpression experiments of *Shh* revealed that ectopic *Shh* could induce floor plate cells in the dorsal neural tube (Echelard *et al.,* 1993; Roelink *et al.,* 1994). Furthermore, it has been reported that ectopic *Shh* represses normal growth of the tectum, producing dorsally enlarged tegmentum region, in which there are a considerable number of SC1-positive motor neurons with ventral markers such as HNF-3β, Isl-1 and Lim1/2, while the expression of Pax7, the alar plate marker of the pretectum (P1) and tectum, was decreased by ectopic *Shh* (Watanabe and Nakamura, 2000). All these results indicate that ectopic *Shh* may induce a fate change from the alar plate to the basal plate, which is consistent with the idea that Shh acts as a common ventralizing signal along the neural tube. However, in our study, overexpression of *Shh* did not alter the expression domain of *Tcf4*, the alar plate marker of P1 and P2 (Fig. 3E, G), suggesting that the identity of the alar plate is not completely changed after *Shh* overexpression in this study. Otx2 expression was not changed. On the other hand, there were a variety of changes in the expression of chick Dbx1 after Shh overexpression (Fig. 4). Thus, although Shh is usually seen as a common ventralizing signal along the neural tube, based on our data, the regulation effect of Shh may be dependent on the specificity of genes, differential development stages and regions. It seems still necessary to examine more markers to give a conclusion about the specificity of ectopically induced cells expressing *Lhx1* in P1 by Shh.

We also showed that the activated form of Gli2 (Gli2–ΔN2)

induced ectopic *Lhx1* expression in small patches in the P1 and mesencephalon, while *Lhx1* expression in P1 on the experimental side was decreased after electroporation of the repressor form of Gli2 (Gli2–ΔC4). This is consistent with the results from the transgenic mouse experiments (Sasaki *et al.,* 1999). These results support the idea that *Lhx1* is induced by the Shh signal through activation of transcription factor Gli2, and imply that *Lhx1* is one of the downstream genes of Shh. However, whether this induction is direct or indirect remains to be determined by detailed analyses of the *Lhx1* cis-regulatory regions. Unexpectedly, Gli1 did not show any effect on expression of *Lhx1*, which may be due to the differential involvement of Gli factors in Shh signaling. This result is consistent with the idea that Gli factors have preference in the property of Hh target gene regulation (Ruiz i Altaba, 1998, 1999; Persson *et al.,* 2002; Karlstrom *et al.,* 2003; Hashimoto-Torii *et al.,* 2003).

Lhx9 *is up-regulated by overexpression of* Wnt3a *and* Fgf15, *and down-regulated by misexpression of* Shh

Three classes of secreted factors have been implicated as candidate signals that specify the fate of dorsal neural cells. These are secreted proteins of the FGF, WNT, and TGFβ families. Members of these families of inductive factors are expressed in or adjacent to the lateral neural plate or dorsal neural tube, consistent with a role for these proteins in specification and/or proliferation of dorsal neural cells. Wnt expression is induced by BMPs (Dickinson *et al.,* 1995). Although the emerging evidence suggests that BMP signaling has a central role in dorsal neural patterning, the complete program of dorsal cell differentiation may necessarily involve the coordinated action of both Wnts and BMPs (Reviewed by Lee and Jessel, 1999). It has also been reported that the ability of FGFs to enhance the generation of neural crest cells appears to be mediated by Wnt family members (LaBonne and Bronner-Fraser, 1998). Previous reports showed that signals from the roof plate are required in the dorsal spinal cord for induction of *Lhx2* and *Lhx9*, which define D1A and D1B interneurons, respectively (Liem *et al.,* 1997; Lee and Jessel, 1999; Lee *et al.,* 2000). We found that *Lhx9* is expressed in the dorsal part of both the caudal neural tube and forebrain, which raised a possibility that the same regulatory mechanisms on *Lhx9* expression may be conserved between the spinal cord and forebrain. In the present study, we investigated the regulatory effects of Wnt3a, Wnt1 and Fgf19 (mouse Fgf15) on *Lhx9* expression. These signaling molecules were all detected in the dorsal part of the caudal diencephalon and spinal cord from early stages. Our data indicate that *Wnt3a* did up-regulate the expression of *Lhx9* most likely through TCF4. *Wnt1*, however, did not show a similar effect. Previous studies strongly suggested redundancy between *Wnt1* and *Wnt3a*, though *Wnt3a* showed a distinct expression pattern in the dorsal P2, implying its unique roles in this region (McMahon and Bradley, 1990; McMahon *et al.,* 1992; Wolda *et al.,* 1993; G. Wong, B. Gavin, and A. McMahon, unpublished data). When this *Wnt1*-expression vector was electroporated into the diencephaon or spinal cord, cell proliferation was increased, consistent with the previous report (Ikeya *et al.,* 1997). Therefore, negative results with Wnt1 in induction of *Lhx9* were not artifacts. Chick *Fgf19* is detected in the dorsal telencephalon and diencephalon from HH stage 14 (Kurose *et al.,* 2004). Moreover, our data indicated that mouse *Fgf15* could up-regulate expression of *Lhx9* in the dorsal diencephalon providing further evidence that mouse *Fgf15* is an orthohog of chick *Fgf19*. Although it remained unknown how the pattern of *Lhx9* is established, we conclude that maintenance of its expression in the dorsal diencephalon is regulated by the signals from the roof plate. We confirmed that *Lhx9* in the dorsal diencephalon is positively regulated by the dorsal signals with a similar mechanism as in the spinal cord. Although Wnt3a and Fgf15 both up-regulated *Lhx9* expression, we could not address the synergism between them. It is not clear what a position relationship between Wnt3a and Fgf15 is involved in regulating the expression of *Lhx9*. So, based on our data, we cannot exclude the possibility that when *Lhx9* responded to one of the two signaling molecules and reached a saturated state, it would not be responsive to another more.

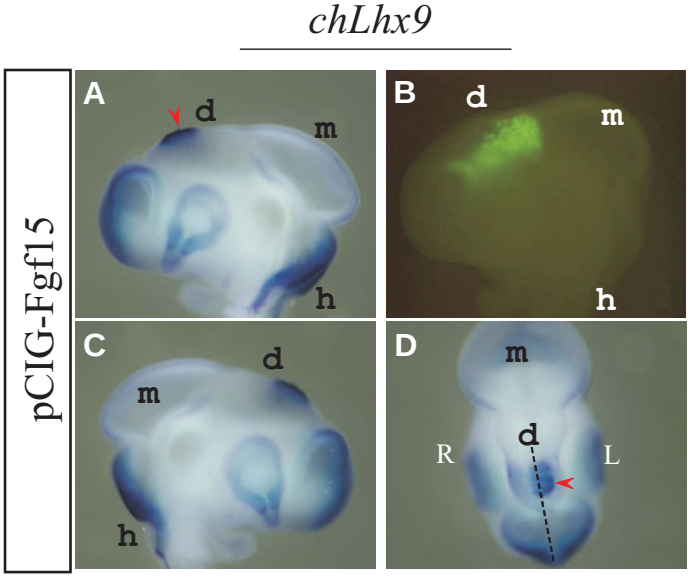


Fig. 8. Up-regulation of *Lhx9* expression by *Fgf15* overexpression. *Embryos were fixed at HH stage 18.* **(A,C)** *Lateral views of experimental and control sides, respectively.* **(B)** *GFP indicates the electroporated domain.* **(D)** *A dorsal view. Electroporation of pCIG-Fgf15 into the developing diencephalon resulted in up-regulation of* Lhx9 *expression in the dorsal P2 (arrowhead in A, D). Note that intensity of the staining was increased on the electroporated side and that the domain of expression was slightly expanded laterally on the same side. Dashed line, dorsal midline. Abbreviations are as in Fig.1.*

The endogenous expression of *Shh* is not detected in the dorsal-most region of the diencephalon. In the midbrain, ectopic *Shh* strongly suppressed expression of several genes crucial for tectum formation, indicating that the fate of the mesencephalic alar plate is changed to that of the basal plate (Watanabe and Nakamura, 2000). Thus, in the present study, we cannot completely exclude the possibility that the down-regulatory effect of Shh on *Lhx9* is a result of the fate change from dorsal to ventral identity due to the misexpression of *Shh* rather than a direct inhibition by *Shh*.

In summary, our data indicate that LIM-homeobox genes, chick *Lhx1* and *Lhx9*, are expressed in the developing diencephalon in localized patterns. Furthermore, these two genes are regulated by the ventral and dorsal signals, probably with similar mechanisms as in the spinal cord. These results provide evidence

that LIM-homeobox genes may play important roles in regionalization of the chick diencephalon under regulation of the signals of positional information.

Materials and Methods

Embryos

Fertilized chicken eggs from Yamagishi farm (Kyoto, Japan) were incubated in a humidified chamber at 39ºC. Embryos were staged according to Hamburger and Hamilton (HH, 1951). For Whole-mount staining, embryos were fixed in 4% formaldehyde in PBS at 4 ºC overnight. They were rinsed twice with PBT (0.1% Tween 20 /PBS) and dehydrated in a graded series of methanol in PBS to store in 100% methanol at -20 ºC.

In situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (Parr *et al.,* 1993). cDNA plasmids for probes were gifts from Drs. Nakamura (chick *Lhx1* and *Pax7*), and Tabin (chick *Shh* and *Tcf4*). For chick *Lhx9* probe, an approximate 640bp fragment was obtained by RT-PCR from E2.5 chick embryos. This fragment was inserted in pBluescript II SK(-) (Stratagene). After linearization, digoxigenin probes were synthesized using the Digoxigenin RNA labeling Kit (Roche). After hybridization, embryos were cryoprotected and cryostat sectioned 14 μm thick in the transversal plane.

In ovo *electroporation*

Expression vectors were transfected to chick embryos by *in ovo* electroporation as previously described (Funahashi *et al.,* 1999). pCIG-Shh, pCIG-Wnt1 and pCIG-Wnt3a expression vectors (in a final concentration at 1.5μg/μl) were electroporated alone, and pCIG-dnTcf4 (1.5μg/μl) was co-electroporated with pCIR-EGFP (0.3μg/μl) to increase the green fluorescence. pCDNA3.1-HisB-Gli2-ΔN2, pCDNA3.1-HisB-Gli2-ΔC4 and pCDNA3.1-HisB-Gli1 (3.0 μg/μl) were cotransfected with pEGFP-N2 (Clontech) (0.5 μg/μl).

DNA solution of 0.1 - 0.2 μl in TE buffer was injected into the neural tube at HH stage 10 - 11 (Hamburger and Hamilton, 1951). A pair of electrodes (0.5 mm diameter, 1.0 mm length and 4 mm distance between the electrodes) was put beside brain vesicles on the vitelline membrane. A rectangular pulse of 25 V, 50 mseconds was charged 5 times with 1 second intervals by a CUY21 electroporator (NEPA GENE). Efficiency of electroporation was monitored by GFP expression under a fluorescence dissection microscope (MZ FL III, Leica). DNA was transfected into the left side unless indicated.

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