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Six3 inactivation causes progressive caudalization and aberrant patterning of the mammalian diencephalon

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The homeobox gene *Six3* represses *Wnt1* transcription. It is also required in the anterior neural plate for the development of the mammalian rostral forebrain. We have now determined that at the 15- to 17-somite stage, the prospective diencephalon is the most-anterior structure in the *Six3*-null brain, and *Wnt1* expression is anteriorly expanded. Consequently, the brain caudalizes, and at the 22- to 24-somite stage, the prospective thalamic territory is the most-anterior structure. At around E11.0, the pretectum replaces this structure. Analysis of *Six3;Wnt1* double-null mice revealed that Six3-mediated repression of *Wnt1* is necessary for the formation of the rostral diencephalon and that Six3 activity is required for the formation of the telencephalon. These results provide insight into the mechanisms that establish anteroposterior identity in the developing mammalian brain.

KEY WORDS: Mouse, *Six3*, *Wnt1*, Forebrain, Diencephalon, Zona limitans intrathalamica (ZLI)

INTRODUCTION

The forebrain arises from the anterior neuroectoderm (ANE) during gastrulation, and different forebrain identities arise within the rostral neural plate (Shimamura and Rubenstein, 1997; Stern, 2002; Wilson and Houart, 2004). Graded posteriorizing signals specify posterior identity to the ANE (Lumsden and Krumlauf, 1996; Rhinn et al., 2005); among others, Fgfs, retinoic acid, BMPs and Wnts have been proposed as posteriorizing signals in this process (Gamse and Silve, 2000; Wilson and Rubenstein, 2000; Yamaguchi, 2001). During vertebrate head development, the level of Wnt activity specifies posterior-to-anterior fates within the neural plate (Niehrs, 1999; Heisenberg et al., 2001; Kiecker and Niehrs, 2001), and rostral forebrain development may require suppression of caudalization signals; otherwise, caudal diencephalic identity will arise (Kudoh et al., 2002; Wilson and Houart, 2004; Stern, 2005). Wnt antagonists secreted by the ANE and adjacent anterior mesendoderm maintain an anterior Wnt signaling-free zone (Niehrs, 1999; Houart et al., 2002).

The homeobox gene *Six3* maintains anterior forebrain identity by repressing *Wnt1* activity (Lagutin et al., 2003). In mice, *Six3* is first expressed in the anterior neural plate at around embryonic day 7.5 (E7.5). Later, *Six3* is expressed in the ANE, presumptive eye field, ventral forebrain, optic cup, tegmentum and the alar plate of the prethalamus (Oliver et al., 1995). Upon interaction with Groucho-related co-repressors, Six3 acts as a transcriptional repressor (Zhu et al., 2002), and in the lens as a transcriptional activator (Liu et al., 2006). Six3 promotes proliferation by antagonizing the replication-initiation inhibitor geminin (Del Bene et al., 2004) and by repressing Bmp4 expression and BMP signaling (Gestri et al., 2005). In humans, *SIX3* mutations have been associated with holoprosoencephaly (Pasquier et al., 2005; Ribeiro et al., 2006).

Six3-null embryos lack the telencephalon and exhibit severe craniofacial abnormalities (Lagutin et al., 2003). In *Six3*-null embryos, normal *Wnt1* expression in the prospective midbrain is upregulated at around the 1- to 2-somite stage and expands anteriorly at the 6- to 8-somite stage (Lagutin et al., 2003). Six3 directly represses *Wnt1* in vivo, and its activity is necessary to maintain a Wnt-free anterior neural identity during early forebrain development (Lagutin et al., 2003).

Once the initial anteroposterior (AP) pattern of the neural plate is established, further local regionalization is required. One signaling center that influences neural plate patterning is the zona limitans intrathalamica (ZLI), located in the boundary between prethalamus and thalamus. In chicken, the mutual repression of *Six3* and *Irx3* may control ZLI positioning (Kobayashi et al., 2002). Later, basal plate signals induce ZLI expansion through the alar plate, and signals from the dorsal diencephalon midline oppose its formation (Larsen et al., 2001; Zeltser, 2005; Vieira and Martinez, 2006). The ZLI expresses *Shh* (Echelard et al., 1993).

To better understand how antagonists of caudalizing signals function during early mammalian forebrain development, how ectopic anterior expansion of Wnt signaling affects rostral forebrain patterning, and which aspects of the *Six3* brain phenotype were caused by abnormal ectopic expansion of *Wnt1* and which were caused by other *Six3* functions, we performed a detailed temporal characterization of the developing *Six3*-null and *Six3;Wnt1* double-null brains.

We found that the abnormal posteriorization of the *Six3*-mutant brain is gradual and starts in the roof plate at around the 3- to 6-somite stage, continuing until around E11.0. At the 22- to 24-somite stage, the prospective prethalamus is replaced by the prospective thalamus and tegmentum. We also determined that the ZLI is defective in the *Six3*-null brain; therefore, as late as E11.0, the prospective thalamus acquires a pretectum identity. Finally, we conclude that Six3-mediated repression of *Wnt1* is required to avoid alar plate posteriorization and to allow ZLI formation, thereby ensuring proper AP patterning of the diencephalon. In addition, *Six3* activity is required for the formation of the telencephalon.

MATERIALS AND METHODS

Mice

Six3$^{+/-}$ mice (Lagutin et al., 2003), *Six3-Cre* mice (Furuta et al., 2000) and *Six3*$^{F/F}$ mice (Liu et al., 2006) were previously described. *CAGG-CreERT2* mice (Hayashi and McMahon, 2002) and *Wnt1*$^{+/-}$ mice (McMahon and

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Bradley, 1990) were provided by A. McMahon (Harvard University, Cambridge, MA). *Six3*$^{F/F}$ mice were crossed to *Six3*$^{+/-}$*;Six3-Cre* and *Six3*$^{+/-}$*;CAGG-CreERT2* mice to produce *Six3*$^{F/-}$*;Six3-Cre* and *Six3*$^{F/-}$*;CAGG-CreERT2* embryos. For the induction of Cre recombination, pregnant dams were injected intraperitoneally with tamoxifen (TM; 1 mg/15 g body weight) dissolved in safflower oil at various embryonic stages. Genotypes were determined by PCR analysis. Wild-type and *Six3*$^{+/-}$ embryos were used as controls. For embryonic staging, the following standardized nomenclature was used: E8.5, 10- to 15-somite stage; E9.0, 15- to 20-somite stage; E9.5, 20- to 25-somite stage; E10.0, 25- to 30-somite stage; E10.5, 30- to 35-somite stage; E11.0, 35- to 40-somite stage.

In situ hybridization and S-Gal staining

S-Gal staining was performed as described (Kishigami et al., 2006). S-Gal-stained embryos were washed in PBS, fixed with 4% paraformaldehyde (PFA) for 1 hour on ice and used for whole-mount in situ hybridization. For whole-mount in situ hybridization analysis, embryos were fixed for 2 hours in 4% PFA on ice and processed as described (Belo et al., 1997). In situ hybridization in sections was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Double in situ hybridization/immunohistochemistry was performed as described (Lavado and Oliver, 2007). Images were obtained on a Leica MZFLIII stereomicroscope equipped with a Hanamatsu C5810 camera.

TUNEL and proliferation assays

TUNEL assay of whole-mount embryos was performed as described (Smith and Cartwright, 1997). TUNEL assay on tissue sections was performed using the ApoTag Plus Peroxidase Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. For proliferation assays, phosphohistone H3 (PH3) immunohistochemistry was performed as described (Lavado and Oliver, 2007). Time-mated female mice were injected with BrdU (40 μg/g body weight, intraperitoneally), and embryos were harvested 1 hour later. Anti-BrdU monoclonal antibody (1:10; BD Biosciences, San Jose, CA) was used. Immunohistochemistry images were acquired with an Axiovert 1.0 microscope (Zeiss, Jena, Germany) equipped with a Progres C14 camera (Jenoptic, Jena, Germany).

RESULTS

Establishment of brain identity is delayed in the anterior *Six3*-null head

Six3-null embryos lack the telencephalon, and around the 6- to 8-somite stage the *Wnt1* expression domain ectopically expands into the ANE, resulting in posteriorization of the mutant roof plate (Lagutin et al., 2003). We have now determined that the expression of the midbrain and diencephalic markers *Otx1* (Simeone et al., 1992a) and *Irx1* (Bosse et al., 1997) is also anteriorly expanded in 6- to 8-somite stage *Six3*-null brains (see Fig. S1A′,B′ in the supplementary material). However, expression of the prethalamic markers *Lhx5* (Sheng et al., 1997), *Arx* (Miura et al., 1997), *Fezf1* and *Fezf2* (Hirata et al., 2004; Hirata et al., 2006a) (see Fig. S1C′-F′ in the supplementary material) suggested that the ventral prospective prethalamus is present in the *Six3*-null brain. Moreover, the reduced expression of *Nkx2.1* (Shimamura et al., 1995) and the absence of *Six6* (Jean et al., 1999) suggested that the anterior hypothalamus is absent or very small at this stage (see Fig. S1G′,H′ in the supplementary material). This is the first evidence that the *Six3*-null brain is abnormally caudalized as early as the 6- to 8-somite stage.

Next, we analyzed the caudalization process during later stages. At around the 16-somite stage, *Irx1* expression was detected in the mesencephalon and caudal diencephalon of the wild-type brain (Fig. 1A), and expression of *Lhx5* was observed in the prethalamus and telencephalon (Fig. 1B). *Arx* was detected in the prethalamus and dorsal telencephalon (Fig. 1C). *Fezf1* and *Fezf2* were detected in the ventral prethalamus and telencephalon (Fig. 1D,E). *Nkx2.1* was detected in the prethalamus and posterior hypothalamus (Fig. 1F), and *Six6* was found in the anterior hypothalamus and Rathke's pouch (Fig. 1G). At this somitic stage, *Irx3* was detected in the mesencephalon, pretectum and thalamus of the wild-type brain (Bosse et al., 1997) (Fig. 1H), and *Tcf4* was observed in the alar plate of the pretectum, thalamus and ventral prethalamus (Cho and Dressler, 1998) (Fig. 1J).

Analysis of these same molecular markers in *Six3*-null littermates detected *Irx1* in a restricted portion of the most-anterodorsal region of the *Six3*-null brain but not in most of the alar plate (Fig. 1A′). By contrast, *Lhx5* was widely expressed in the anterior basal and alar plates (Fig. 1B′) and *Arx* (Fig. 1C′), *Fezf1* (Fig. 1D′) and *Fezf2* (Fig. 1E′) were restricted to a small ventral portion of the *Lhx5*-expression domain. The expression of *Nkx2.1* (Fig. 1F′) and *Six6* (Fig. 1G′) suggested that the anterior hypothalamus is reduced or absent in the *Six3*-null brain. In contrast to the ectopic anterior expansion of *Wnt1*, *Irx1*, *Pax3* or *Otx1* detected at the 6- to 8-somite stage, expression of *Irx3* and *Tcf4* was not expanded, even at the 17-somite stage (Fig. 1H′,J′). These results suggest that at the 17-somite stage, the ventral part of the anterior *Six3*-null brain has a prospective prethalamic identity, and that the *Irx1*-expressing dorsal portion has not yet acquired a prospective thalamic identity.

Analysis of the *Six3*-null brain at later stages revealed graded, anterior expansion of the caudal diencephalic markers *Irx3* (Fig. 1I′) and *Tcf4* (Fig. 1K′ and see Fig. S2 in the supplementary material), indicating that although anterior expansion of *Wnt1* started early, the resulting abnormal posteriorization of the *Six3*-null brain was not complete until the 22- to 24-somite stage. The presence of an *Irx3*- and *Tcf4*-free territory rostral to the posterior diencephalon at the 17-somite stage, and the later anterior expansion of these markers, could have been caused by cell death in the anterior brain territory, and replacement of those cells by posterior diencephalon cells and/or surviving rostral cells acquiring a posterior diencephalic identity.

To analyze these possibilities, TUNEL assay was performed in embryos at the 17-somite stage, when posteriorization of the mutant brain starts. Few apoptotic cells were identified in the rostral diencephalon of two wild-type brains (mean, 9.29/100 cells; s.d., 5.88) (Fig. 1M), and the mean number of apoptotic cells was significantly higher in the anterior-most region of the *Six3*-null brain (mean, 53.73/100 cells; s.d., 10.41; $P=4.057\times10^{-7}$) (Fig. 1M′,N). The percentage of apoptotic cells was reduced in the anterior *Six3*-null brain at the 21-somite stage (mean, 6.58/100 cells; s.d., 3.08; $n=3$) (Fig. 1N′). Analysis of cell proliferation using PH3 immunostaining revealed no differences between the wild-type anterior diencephalon (mean, 9.17/100 cells; s.d., 0.62) and the *Six3*-null rostral brain (mean, 11.51/100 cells; s.d., 1.46; $P=0.0146$) (data not shown); also, no differences were detected in the posterior diencephalon (data not shown).

These results indicated that abnormal caudalization of the *Six3*-null rostral brain is a gradual process that starts at the 2- to 3-somite stage in the ANE, continues into the roof plate a few somites later, and posteriorizes the alar plate by the 22- to 24-somite stage. The alar plate (prospective pretectum and thalamus) was abnormally displaced toward the most-anterior region of the mutant brain, and the establishment of brain territory identities was delayed in that region. The increase in cell death detected in the 17-somite stage *Six3*-null brain probably contributed to the gradual posteriorization identified at the 17- to 24-somite stage. Furthermore, the lack of proliferation differences between wild-type and mutant posterior diencephalon suggests that cell fate change could also contribute to caudalization of the *Six3*-null brain at these stages.

Prospective thalamus is the most-anterior structure of the alar plate in the 22- to 24-somite stage *Six3*-null brain

We analyzed the expression of additional alar and basal plate markers at later stages. At the 22- to 24-somite stage, *Wnt3a* (Parr et al., 1993) was expressed in the mesencephalon, roof and alar plates of the thalamus and roof plate of the prethalamus (Fig. 2A). *Fgf15* (McWhirter et al., 1997) was expressed in the posterior mesencephalon and alar plate of the thalamus (Fig. 2B) and *Otx1* was localized in the mesencephalon and alar plate of the diencephalon (Fig. 2C). Thus, in the 22- to 24-somite stage *Six3*-null littermates, the prospective thalamus (Fig. 2A′-C′) is the most-anterior structure in the alar plate. These findings made us question whether these alterations affect the prethalamus.

In wild-type controls, *Fezf1* and *Fezf2* are expressed in the prethalamus (Fig. 2D), and *Dlx2* (Porteus et al., 1991) in the ventral-most part of the prethalamic alar plate (Fig. 2E). In *Six3*-null littermates, the expression of *Fezf1* (Fig. 2D′) and *Fezf2* (data not shown) was greatly reduced, and few *Dlx2*-expressing cells were detected (Fig. 2E′). We conclude that only a small portion of the ventral prethalamus is present in the *Six3*-null brain at this stage.

Next, we analyzed how this posteriorized phenotype affects the mutant basal plate. *Nkx2.2* is normally expressed at the boundary between the alar and basal plates (Fig. 2F) (Shimamura et al., 1995). In the *Six3*-null brain, its expression in this region was mostly unaffected (Fig. 2F′), the exception being the truncated anterior-most domain (Fig. 2F′). This finding indicated that an alar-basal boundary is present in the mutant head. However, truncated expression of *Nkx2.2* in the anterior basal plate suggested that the hypothalamus is defective. This proposal was supported by the observation that *Bmp4* (Jones et al., 1991), a marker for the basal plate of the posterior hypothalamus (Fig. 2G), was not detected in the *Six3*-null brain at any stage analyzed (Fig. 2G′ and data not shown).

Normally, *Wnt1* is expressed in the basal plate at the level of the cephalic flexure (Fig. 2H) (Prakash et al., 2006); its expression was also expanded anteriorly in the mutant brain (Fig. 2H′). *Ngn2* (also known as *Neurog2* – Mouse Genome Informatics) and *Foxa2* are normally expressed in the ventral neural tube, from the caudal spinal cord to the tegmentum (Fig. 2I,J) (Gradwohl et al., 1996; Sasaki and Hogan, 1993). In the *Six3*-null brain, their expression in the basal plate was anteriorly expanded (Fig. 2I′,J′). Together, these results indicated that the tegmentum expands into the anterior basal plate of the *Six3*-null brain at the 22- to 24-somite stage. Moreover, the hypothalamic

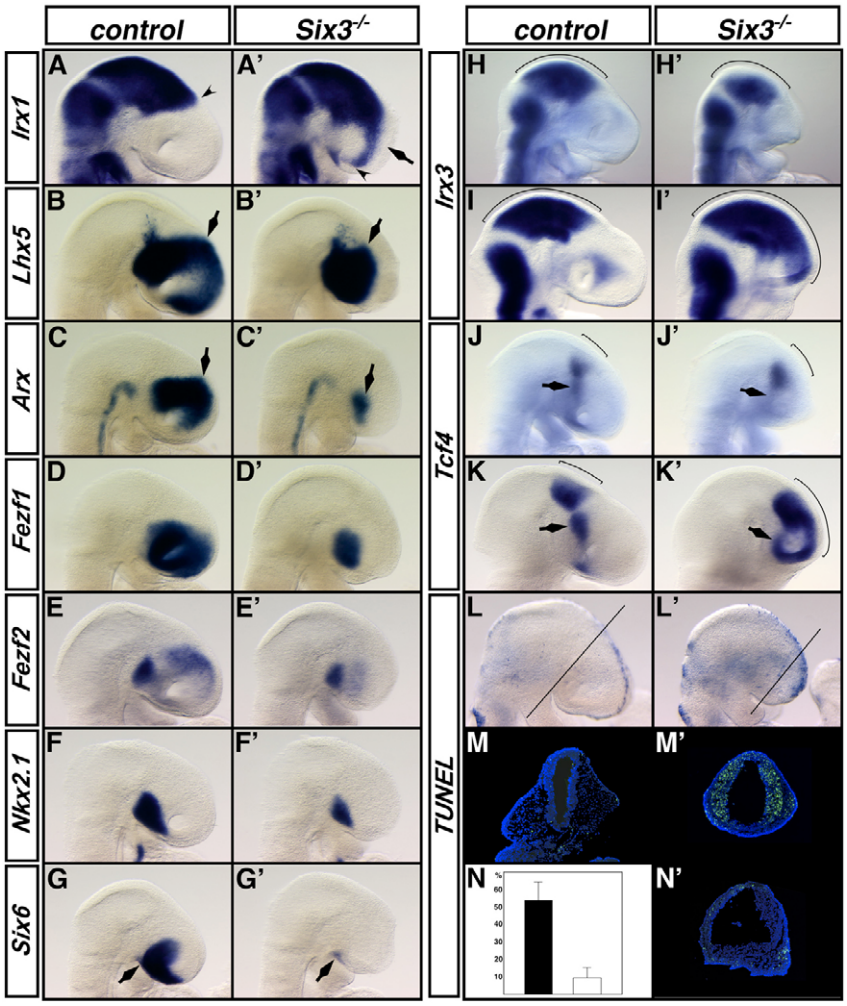

Fig. 1. The anterior region of the *Six3*-null mouse brain progressively caudalizes during development. (**A-G′**) At the 15- to 17-somite stage, (A) *Irx1* is expressed in the mesencephalon, pretectum and thalamus of the wild-type brain, (B) *Lhx5* in the prethalamus and the telencephalon, (C) *Arx* in the prethalamus and dorsal telencephalon, (D) *Fezf1* and (E) *Fezf2* in the ventral prethalamus and telencephalon, (F) *Nkx2.1* in prethalamus and hypothalamus, and *Six6* (G) in the Rathke's pouch (arrow) and anterior hypothalamus. (**H-K′**) At the 15- to 22-somite stages, *Irx3* is normally expressed in the mesencephalon, pretectum and thalamus (H,I). At the 19- to 22-somite stages, *Tcf4* is normally expressed in the pretectum, thalamus (bracket) and ventral prethalamus (arrow) (J,K). At these somitic stages, the anterior-most part of the *Six3*-null brain has not fully posteriorized, as indicated by the anteriorly expanded *Irx1* expression (A′, arrowhead) and lack thereof in the dorsal brain (arrow), the presence of *Lhx5* (B′) and the absence of *Irx3* and *Tcf4* expression (H′,J′, brackets). Reduced *Nkx2.1* expression (F′) and the absence of *Six6* (G′) confirmed the absence or reduction of the hypothalamus in the *Six3*-null brain. Residual *Six6* expression in surface ectoderm at the 15-somite stage suggested the presence of the Rathke's pouch region (arrow); however, no such structure is present at later stages. Later, the alar plate of the *Six3*-null brain gradually caudalizes, and at the 22- to 23-somite stage, the expression of *Irx3* and *Tcf4* (I′,K′, brackets) is anteriorly expanded. (**L-N′**) At the 17-somite stage, an abnormally large number of TUNEL$^+$ cells were seen in the anterior region of the *Six3*$^{-/-}$ brain (L′), as compared with wild type (L). Similar results were observed when the TUNEL assay was performed on sections of wild-type (M) and *Six3*$^{-/-}$ (M′) brains. The plane of the tissue sections shown in M,M′ is indicated by a line in L,L′. A drastic reduction in the number of apoptotic cells was observed in the anterior region of the *Six3*-null brain between the 17- and 21-somite stages (M′,N′). (N) Bar chart showing the number of TUNEL$^+$ cells per 100 DAPI$^+$ cells at the 17-somite stage; white bar, wild-type; black bar, *Six3*-null. Arrows in B,C,D indicate the dorsal limit of expression. Anterior is to the right.

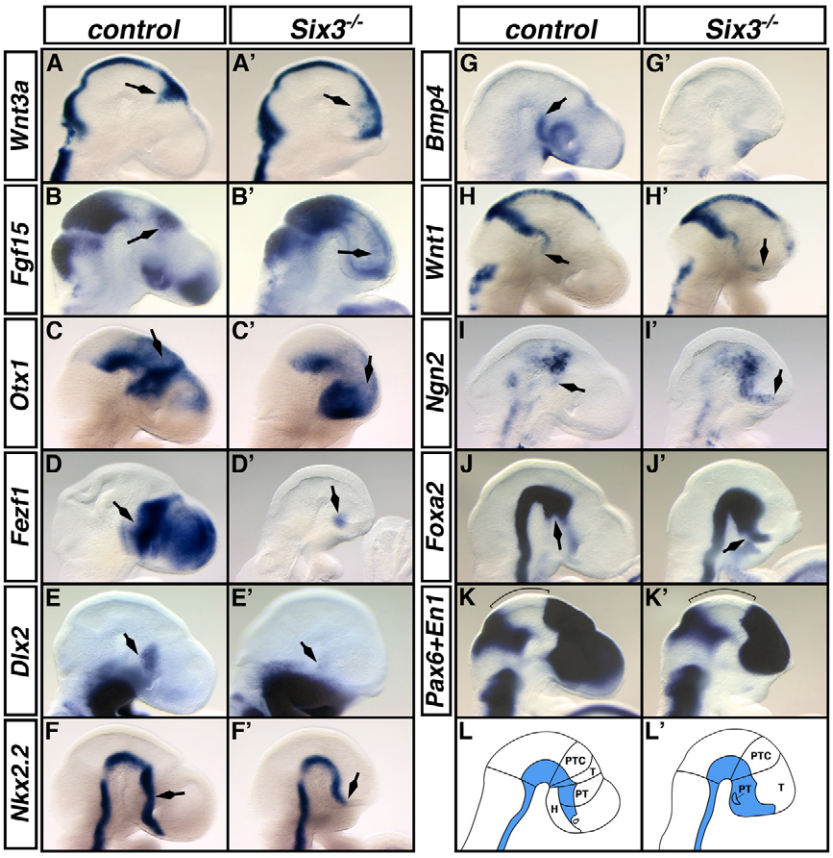

Fig. 2. The prospective thalamus is the most anterior structure of the *Six3*-null brain at the 22- to 24-somite stage. (**A**) *Wtn3a* is normally detected in the roof and alar plate (arrow) of the thalamus at the 22- to 24-somite stage; *Fgf15* (**B**) and *Otx1* (**C**) are localized in the alar plate of the thalamus (arrows) and prethalamus. In *Six3*-null mouse embryos, the prospective thalamus is located in the anterior region of the brain, as indicated by the expression of *Wnt3a* (arrow, **A′**), *Fgf15* (arrow, **B′**) and *Otx1* (arrow, **C′**). The prospective prethalamus, as shown by *Fezf1* (**D**) and *Dlx2* (**E**) (arrows), is reduced in the *Six3*-null brain (arrows, **D′**,**E′**). The alar plate-basal plate border, as indicated by *Nkx2.2* expression (**F**), is also reduced (arrow, **F′**; the arrow in F shows the equivalent region to F′). *Bmp4* is normally expressed in the basal plate of the posterior hypothalamus (arrow, **G**) and is absent in the *Six3*-null brain (**G′**). In the wild-type basal plate, *Wnt1* is expressed in the ventral midline (**H**), and *Ngn2* (**I**) and *Foxa2* (**J**) are expressed more laterally in the tegmentum. The expression of these genes is expanded anteriorly in the *Six3*-null brain (**H′**,**I′**,**J′**). Arrows in H-J′ indicate the most-anterior limit of the expression domain. The size of the mesencephalon, as indicated by the *Fgf15* expression domain (B), and the region between the *Pax6*$^{+}$ and *En1*$^{+}$ territories (**K**, bracket), are normal in the *Six3*-null brain (B′,**K′**). Schemes of the 22- to 24-somite stage wild-type (**L**) and *Six3*-null (**L′**) brain. The pretectum, thalamus and prethalamus are located dorsally to the hypothalamus in the wild-type brain. In the *Six3*-null brain, the thalamus is the most anterior structure, and only a small portion of the prethalamus remains at this stage. The basal plate is shown in blue. Lines delimit the prospective regions. Anterior is to the right. HY, hypothalamus; PT, prethalamus; PTC, pretectum; T, thalamus.

region was reduced or absent, possibly owing to this expansion. As indicated by the expression of *Fgf15*, *En1* and *Pax6*, no alterations were detected in the size or positioning of the mesencephalon in the *Six3*-null brain (Fig. 2B′,K′). In conclusion, the mutant alar plate is posteriorized at the 22- to 24-somite stage, and the prospective thalamus is the most-anterior alar plate boundary (Fig. 2L′). In the basal plate, the tegmentum and a severely reduced ventral prethalamus are the most-anterior structures remaining (Fig. 2L′).

The prospective thalamus is replaced by an expanded pretectum in the *Six3*-null brain during late development

To determine whether caudalization of the *Six3*-null brain continues beyond the 22- to 24-somite stage, we analyzed the expression of markers for the thalamus and pretectum at later stages. At E14.5, *Tcf4* is normally expressed in the pretectum and thalamus (Fig. 3B). In the *Six3*-null brain, *Tcf4* expression was displaced toward the most-anterodorsal region (Fig. 3B′). At E13.5, *Lhx2* was detected in the thalamus and at the tegmentum-pretectum boundary (Fig. 3C) (Nakagawa and O'Leary, 2001). *Lhx2* thalamic expression was not detected in the anterior territory of the *Six3*-null brain (Fig. 3C′ and see Fig. S3F in the supplementary material); thus, thalamic formation was probably arrested in the mutant brain at these later stages. Expression of other thalamic markers, such as *Gbx2*, *Ngn2* (Nakagawa and O'Leary, 2001) and *Prox1* (Lavado and Oliver, 2007), was not detected in this brain region (see Fig. S4 in the supplementary material). These results suggested that the prospective thalamus identified in the *Six3*-null brain during early development was later replaced by the pretectum.

To confirm this possibility, we analyzed the expression of additional markers. In the E14.5 wild-type brain, the pretectum marker *Lim1* (also known as *Lhx1* – Mouse Genome Informatics) is expressed in the tegmentum, pretectum and prethalamus (Fig. 3D) (Fujii et al., 1994), and *Ebf1* in the rostral pretectum (Fig. 3E) (Garel et al., 1997). In the *Six3*-null littermates, *Lim1* (Fig. 3D′) and *Ebf1* (Fig. 3E′) were found in the anterodorsal region, and the

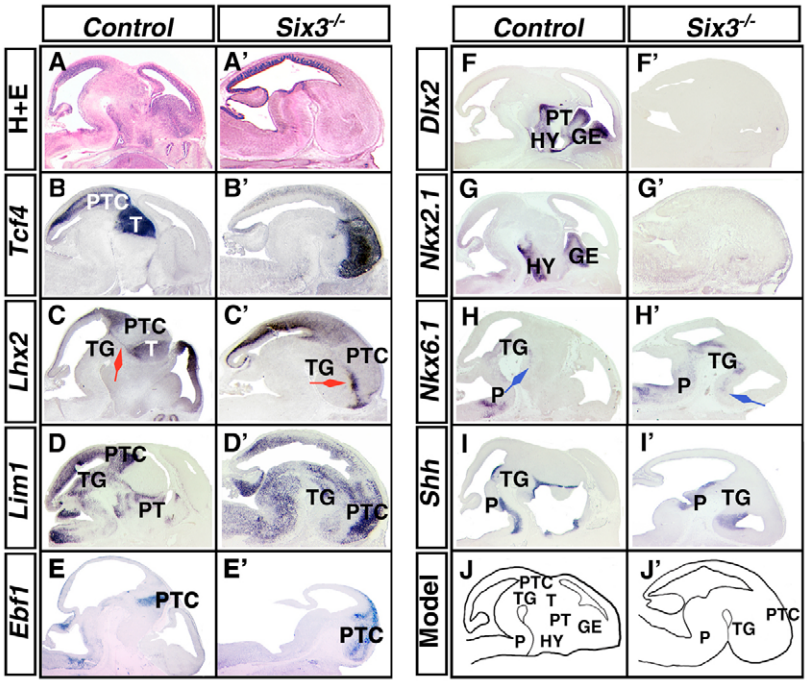

Fig. 3. The prospective thalamus is replaced by the pretectum in the *Six3*-null brain. Hematoxylin and Eosin staining of sagittal sections from E14.5 wild-type (**A**) and *Six3*-null (**A′**) mouse brains. At E14.5, *Tcf4* is expressed in the pretectum and thalamus of wild-type embryos (**B**) and in the anterior-most region of the *Six3*-null brain (**B′**). At E13.5, *Lhx2* is normally expressed in the thalamus (**C**), but is absent from the *Six3*-null brain (**C′**). *Lhx2* is also expressed between the tegmentum and pretectum of the wild-type brain (arrow, C,C′). At E14.5, *Lim1* is normally expressed in the pretectum (**D**), but is shifted anteriorly in the *Six3*-null brain (**D′**). *Ebf1* is expressed in the anterior pretectum of wild-type embryos (**E**) and in the anterior region of the *Six3*-null brain (**E′**). As indicated by the absence of *Dxl2* (**F′**) and *Nkx2.1* (**G′**) expression at E14.5, the prethalamus (**F**) and the hypothalamus (**G**) were not present in the *Six3*-null brain. Instead, as indicated by *Nkx6.1* expression at E12.5, the tegmentum is located in the ventral region of the mutant brain (**H′**, arrows point to the limit of *Nkx6.1* expression; compare with **H**). The ZLI, as revealed by *Shh* expression at E12.5, is not present in the *Six3*-null brain (**I′**; compare with **I**). Therefore, instead of the thalamus (**J**), the pretectum and the tegmentum are present in the anterior region of the *Six3*-null brain (**J′**). Anterior is to the right. GE, ganglionic eminence; HY, hypothalamus; P, pons; PT, prethalamus; PTC, pretectum; T, thalamus; TG, tegmentum.

subcomissural organ was enlarged (see Fig. S5 in the supplementary material). These results confirmed that at later stages, the *Six3*-null alar plate posteriorizes further, and the anterior pretectum replaces the prospective thalamus.

To determine the developmental stage at which the anterior mutant brain acquires pretectum identity, we analyzed the expression of *Lim1*. Normally, *Lim1* expression in the pretectum starts at E10.0; however, it was not detected in the anterior region of the mutant brain until E11.0 (see Fig. S6 in the supplementary material). The lack of obvious alterations in proliferation or cell death in the *Six3*-null brain at these later stages (see Figs S7, S8 in the supplementary material), suggested that the prospective thalamus was transformed into a pretectum.

In addition, the lack of expression of the prethalamic and hypothalamic markers *Dlx2* and *Nkx2.1* (Rinkwitz-Brandt et al., 1995) (Fig. 3F′,G′) at E14.5 confirmed that the hypothalamus is not present in the mutant brain at this stage. As shown by *Lim1* (Fig. 3D′) and *Nkx6.1* (Fig. 3H′) (Qiu et al., 1998) expression, the tegmentum is the most-anterior ventral structure in the basal plate of the *Six3*-null brain. A scheme representing the mutant brain is shown in Fig. 3J′.

Six3 activity is not required for positioning or expansion of the ZLI

As described above, the caudalization of the anterior mutant diencephalon appears to be caused by the gradual replacement of the prospective thalamus by the pretectum. Abnormal anterior expansion of the pretectum has been reported in animal models in which signaling from the ZLI is defective (Hirata et al., 2006b).

In chicken, *Six3* activity is required at the neural plate stage to position the ZLI (Kobayashi et al., 2002); however, in the 30- to 35-somite stage *Six3*-null mouse brain, the *Shh*-expressing ZLI primordium was positioned normally (see Fig. S9 in the supplementary material). Therefore, *Six3* does not mediate the positioning of the ZLI in mice. Whether later ZLI signaling was defective and promoted caudalization of the anterior region of the mutant brain cannot be ruled out.

Normally, ZLI expansion starts in a region between the thalamus and prethalamus at around E9.5 (Shimamura et al., 1995). As shown above, at around E9.5, the expression of prospective thalamic markers, such as *Tcf4*, *Wnt3a* and *Fgf15*, was abnormally expanded in the *Six3*-null brain prior to ZLI expansion; this phenotypic alteration could have affected the competence of the ZLI-forming territory.

To determine whether the ZLI expanded dorsally, we analyzed *Shh* expression at E12.5 (Echelard et al., 1993). *Shh* expression was detected in the wild-type ZLI (Fig. 3I), but not in the corresponding mutant territory (Fig. 3I′). Moreover, the expression of other genes normally detected in or around the ZLI (e.g. *Lim1*, *Nxk2.2*) was also absent (Fig. 3D′ and data not shown). These data demonstrate that the ZLI is not present in the E12.5 *Six3*-null brain.

Although the lack of ZLI was probably caused by the abnormal posteriorization of the rostral mutant brain, we cannot exclude the possibility that *Six3* activity is also required to maintain and/or expand *Shh* expression through the alar plate. To investigate these alternative possibilities, we used a $Six3^{F/F}$ conditional mouse strain (Liu et al., 2006) to selectively delete *Six3* at several time points before or during dorsal ZLI expansion. *Six3-Cre* (Furuta et al., 2000) or the ubiquitous TM-inducible *CAGG-CreERT2* strain (Hayashi and McMahon, 2002) were used to delete *Six3* prior to ZLI expansion (E9.0 and E9.5). During ZLI expansion, *Six3* was deleted by administering TM at E10.5. *Six3* conditional-mutant embryos were analyzed at E12.5. *Six3* activity was efficiently deleted from most brain regions (Fig. 4) and the telencephalon was severely reduced when *Six3* was deleted before E10.5 (Fig. 4F-O).

At E12.5, the expression of *Shh*, *Lim1* and *Ngn2* is normally detected at the prethalamus-thalamus boundary (Fig. 4B-D). *Shh* expression revealed the position of the ZLI (Fig. 4B); *Lim1* expression revealed the pretectum (Fig. 4C); *Ngn2* expression, the thalamus (Fig. 4D); and *Dlx2* expression, the prethalamus (Fig. 4E). No obvious alterations in the ZLI or AP patterning of the diencephalon were identified when *Six3* activity was conditionally deleted using any of the strategies described (Fig. 4G-J,L-O,Q-T). These results support the hypothesis that *Six3* activity is not directly required for the dorsal expansion or maintenance of the ZLI, and suggest that posteriorization of the alar plate establishes a non-permissive territory for the expansion of *Shh* expression, which arrests ZLI formation. During later development, this arrest affects the induction of thalamic fate in the *Six3*-null brain.

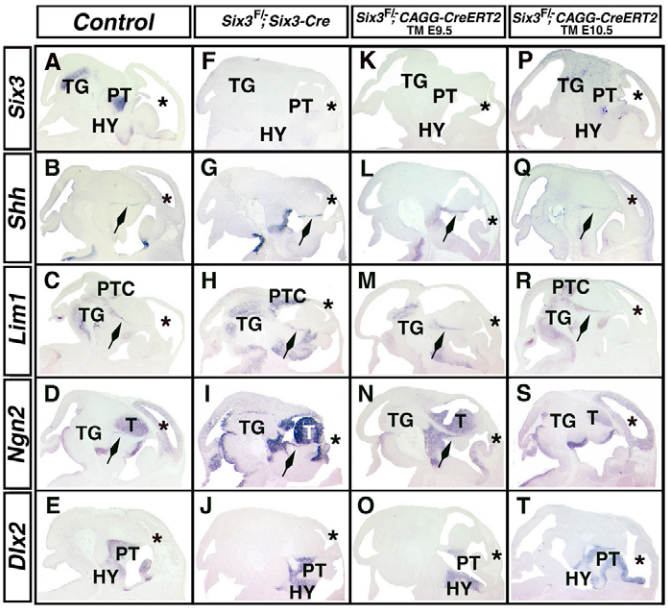

Fig. 4. *Six3* is not necessary for the expansion or maintenance of the ZLI. (**A**) At E12.5, *Six3* is normally expressed in the tegmentum, prethalamus and hypothalamus. (**B-D**) *Shh*, *Lim1* and *Ngn2* (arrows in B,C and D, respectively) expression reveal the location of the ZLI. *Lim1* is also expressed in the pretectum and *Ngn2* in the thalamus. (**E**) *Dlx2* is another marker for the prethalamus. Similar analyses were performed in $Six3^{F/-}$;*Six3-Cre* (**F-J**), $Six3^{F/-}$;*CAGG-CreERT2* (TM-induced at E9.5; **K-O**) and $Six3^{F/-}$;*CAGG-CreERT2* brains (TM induced at E10.5; **P-T**). A similar efficiency of *Six3* deletion was found in all three genotypes (F,K,P). As indicated by the expression of *Shh* (G,L,Q), *Lim1* (H,M,R) and *Ngn2* (I,N), the ZLI (arrows) was formed normally in these mutant mouse embryos. The thalamus, as indicated by *Ngn2* expression (I,N,S), and the prethalamus, as shown by *Dlx2* staining (J,O,T), were present in all of the conditional mutants. Asterisks indicate the telencephalon. Anterior is to the right. HY, hypothalamus; PT, prethalamus; PTC, pretectum; T, thalamus; TG, tegmentum.

Six3 is required for telencephalon formation

During normal forebrain development, Six3 directly represses *Wnt1* expression in the ANE fated to become forebrain (Lagutin et al., 2003). However, we could not discern the aspects of the brain phenotype caused secondarily by the abnormal ectopic expansion of *Wnt1* from those caused by other *Six3* functions in this process. To discriminate between these possibilities, we generated *Six3;Wnt1* double-null embryos. Similar to each single mutant (McMahon and Bradley, 1990; McMahon et al., 1992; Lagutin et al., 2003), $Six3^{-/-}$;$Wnt1^{-/-}$ newborn pups died at birth. Like $Six3^{-/-}$ newborns, $Six3^{-/-}$;$Wnt1^{-/-}$ pups had no eyes and exhibited severe craniofacial abnormalities (data not shown). Similar to $Wnt1^{-/-}$ brains, $Six3^{-/-}$;$Wnt1^{-/-}$ brains lacked the cerebellum and colliculus and had a severely reduced midbrain (Fig. 6A′).

Foxg1 (Tao and Lai, 1992) expression was detected in the telencephalon of E10.5 control (Fig. 5A) and $Wnt1^{-/-}$ embryos (Fig. 5A′). At this stage, *Emx1* (Simeone et al., 1992b) was observed in the dorsal telencephalon of wild-type (Fig. 5B) and $Wnt1^{-/-}$ (Fig. 5B′) embryos, and *Tbr1* (Bulfone et al., 1995) was found in the telencephalon and eminentia thalami of control (Fig. 5C) and $Wnt1^{-/-}$ (Fig. 5C′) embryos. However, their expression was not detected in the prospective telencephalic region of *Six3*-null (Fig. 5A″,B″,C″) or $Six3^{-/-}$;$Wnt1^{-/-}$ brains (Fig. 5A‴,B‴,C‴). The few *Tbr1*-expressing cells found in the ventral part of the $Six3^{-/-}$;$Wnt1^{-/-}$ brain (Fig. 5C‴, arrowhead) probably correspond to the eminentia thalami, a diencephalic structure present in the $Six3^{-/-}$;$Wnt1^{-/-}$ mutant brain (data not shown). These results demonstrate that *Six3* activity is required for the formation of the mammalian telencephalon.

The posteriorization of the *Six3*-null diencephalon is *Wnt1*-dependent

Additional analyses of $Six3^{-/-}$;$Wnt1^{-/-}$ embryos were performed to determine whether the prospective thalamus was anteriorly expanded and the prethalamus was lost. Unlike the E10.0 *Six3*-null brain, $Wnt1^{-/-}$ brains showed no ectopic expansion of *Irx1* or *Tcf4* expression (Fig. 5D′,E′). The *Irx1*- and *Tcf4*-free anterior brain territory (Fig. 5D‴,E‴) indicated that the pretectum and prospective thalamus were not anteriorly expanded into the anterior alar plate of the $Six3^{-/-}$;$Wnt1^{-/-}$ brain. Therefore, the *Irx1*- and *Tcf4*-free anterior brain territory most likely corresponds to the prethalamus. This possibility was confirmed by the expression of the prethalamic marker *Fezf1* (Fig. 5F‴). These results confirm that the absence of Six3-mediated *Wnt1* repression causes the posteriorization of the alar plate of the *Six3*-null brain. A schematic representation of each of these brains is shown in Fig. 5G-G‴.

Six3 repression of *Wnt1* activity is required for proper AP patterning of the diencephalon

We examined whether in the non-caudalized diencephalon of the $Six3^{-/-}$;$Wnt1^{-/-}$ brains, the *Shh* expression domain in the ZLI and the formation of the thalamus and prethalamus were normal. The lack of ZLI in E12.5 *Six3*-null embryos (Fig. 3I′) was rescued in the $Six3^{-/-}$;$Wnt1^{-/-}$ brain (Fig. 6B′). In addition, the thalamus, as indicated by *Lhx2* and *Tcf4* expression (Fig. 6C′,D′), and prethalamus, as shown by *BF-2* (also known as *Foxd1* – Mouse Genome Informatics) (Hatini et al., 1994) and *Dlx2* expression (Fig.

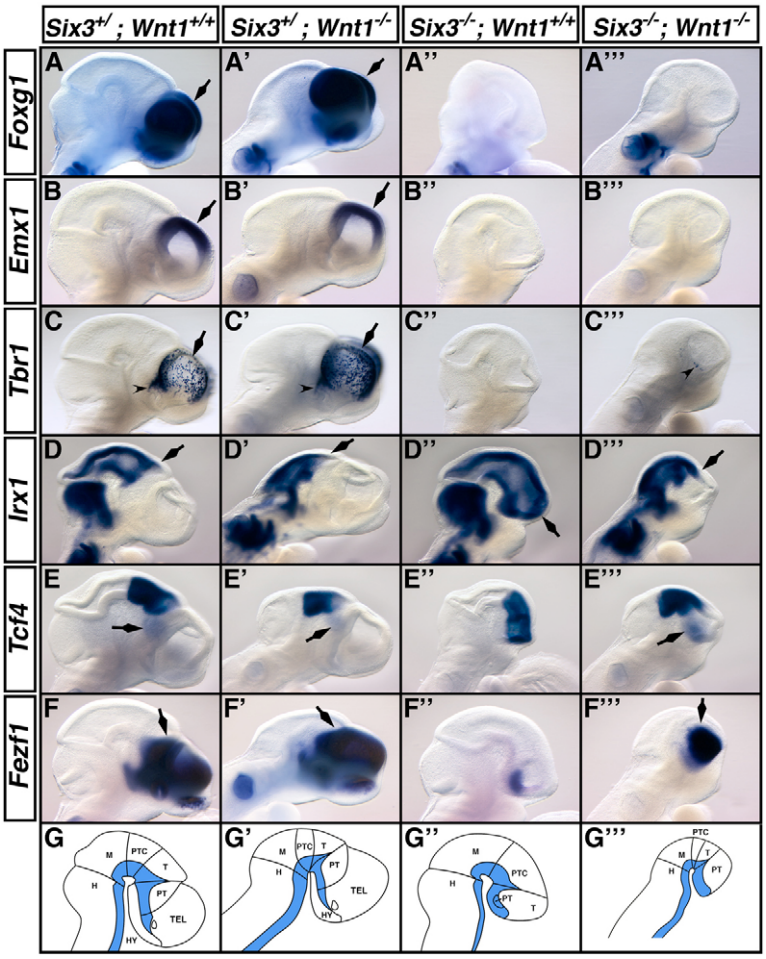

Fig. 5. Prethalamus but not telencephalon is rescued in *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ embryos. *Foxg1* is expressed in the telencephalon of wild-type (**A**) and *Wnt1*$^{-/-}$ (**A′**) mouse brains at E10.5. No telencephalic expression of *Foxg1* was observed in *Six3*$^{-/-}$ (**A″**) or *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brains (**A‴**). *Emx1* is expressed in the dorsal telencephalon of wild-type (arrow, **B**) and *Wnt1*$^{-/-}$ (arrow, **B′**) brains. *Tbr1* is expressed in the telencephalon (arrow) and eminentia thalami (arrowhead) of wild-type (**C**) and *Wnt1*$^{-/-}$ (**C′**) brains. No telencephalic expression of *Emx1* and *Tbr1* was observed in the *Six3*$^{-/-}$ (**B″**,**C″**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (**B‴**,**C‴**) brains. *Irx1* is expressed in the alar plate of the thalamus (arrow) of wild-type (**D**), *Wnt1*$^{-/-}$ (**D′**) and *Six3*-null (**D″**) brains at E10.5. In the *Six3*-null brain (D″), *Irx1* expression expands anteriorly; this ectopic expansion was rescued in the *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain (**D‴**). Similar results were obtained with *Tcf4*, a gene that is expressed in the alar plate of the pretectum and in the thalamus of wild-type (**E**), *Wnt1*$^{-/-}$ (**E′**), *Six3*$^{-/-}$ (**E″**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ embryos (**E‴**). The prethalamus (arrow), as indicated by *Fezf1* expression in the wild-type (**F**) and *Wnt1*$^{-/-}$ (**F′**) brain, is extremely reduced in the *Six3*-null brain (**F″**); however, it appears normal in the *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain (**F‴**). A model for the *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain at this stage is shown (**G‴**). In the *Wnt1*$^{-/-}$ brain (**G′**), the mesencephalon is smaller than in the wild-type brain (**G**). The prospective thalamus is the most anterior structure in the *Six3*-null brain (**G″**), and the prethalamus remains in the *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain (G‴) at this stage. The basal plate is indicated in blue. Lines delimit the prospective regions. Anterior is to the right. H, hindbrain; HY, hypothalamus; M, mesencephalon; PT, prethalamus; PTC, pretectum; T, thalamus; TEL, telencephalon.

6E′,F′), were rescued in the E14.5 *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain. A partial rescue of these structures was observed in E14.5 *Six3*$^{-/-}$;*Wnt1*$^{+/-}$ brains (see Fig. S10 in the supplementary material).

These results suggest that the posteriorization of the anterior alar plate in the *Six3*-null brain is a *Wnt1*-dependent process that interferes with the normal dorsal expansion of the ZLI. Therefore, nearly all of the prospective prethalamic territory was lost and replaced by the thalamus at the 22- to 24-somite stage; by E11.0, the territory was transformed into pretectum. Finally, as revealed by *Nkx2.1* expression, part of the posterior hypothalamus (most likely the dorsomedial hypothalamic nucleus, data not shown) was present in the *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brain at E14.5 (Fig. 6G′). This result suggests that *Six3* function is also required for the formation of the anterior hypothalamus.

DISCUSSION

The developing *Six3*-null brain progressively caudalizes

Early AP patterning of the forebrain partially results from a balance between caudalizing signals and their anterior antagonists (Lumsden and Krumlauf, 1996; Kudoh et al., 2002; Wilson and Houart, 2004; Rhinn et al., 2005). Wnt signaling molecules comprise a well-characterized family of caudalizing factors (Niehrs, 1999; Yamaguchi, 2001; Houart et al., 2002). For example, in chicken and *Xenopus* embryos, Wnt signaling inhibits anterior forebrain markers and promotes posterior fates (Kiecker and Niehrs, 2001; Braun et al., 2003). In zebrafish, forebrain formation is affected in *headless* (*hdl*) mutants, in which *tcf3* (also known as *tcf7l1a* – ZFIN), a component of the Wnt pathway, is mutated (Kim et al., 2000), and loss of *wnt8* function results in zebrafish embryos with a reduced midbrain and a larger forebrain (Lekven et al., 2001). *Six3* is expressed in the ANE during early development and, as shown in chicken and mouse, it represses *Wnt1* signaling in the developing brain (Braun et al., 2003; Lagutin et al., 2003). Similar to *Six3*-null mouse embryos (Lagutin et al., 2003), in *hdl*-mutant zebrafish embryos, Wnt signaling ectopically expands, and *six3* expression is drastically reduced (Kim et al., 2000).

In mice, the lack of *Six3* activity results in ectopic anterior expansion of *Wnt1* expression in the developing brain and, similar to zebrafish, frog and chicken embryos, the roof plate posteriorizes (Lagutin et al., 2003). Data from these organisms lead to the proposal that telencephalic development requires suppression of Wnt signaling from the anterior region; defective suppression results in the prospective forebrain acquiring a more-posterior diencephalic identity.

Here, we determined that the anterior part of the *Six3*-null mouse brain gradually caudalizes; this process starts in the ANE, continues later into the roof and alar plates, and ends once the prospective thalamus is replaced by the pretectum at around E11.0. We also determined that the ectopic anterior expansion of *Irx3* and *Tcf4* expression is a gradual process that is not completed in the alar plate until the 22- to 24-somite stage. These data indicate that although the ectopic anterior expansion of *Wnt1* expression starts at an early somitic stage, the resulting abnormal posteriorization of the *Six3*-null brain is delayed. A possible explanation for this delay is that the anterior-most mutant brain cannot respond to the ectopic Wnt signaling until later embryonic stages.

The lack of telencephalon and eyes reported in medaka fish after *Six3* inactivation using morpholinos has been associated with increased cell death in the ANE of late-gastrula embryos (Carl et al., 2002). The lack of telencephalon in the *Six3*-null mouse brain was detected much earlier than the 17-somite stage, when we observed increased cell death in the anterior brain. Therefore, in mammals, cell death is not responsible for the lack of telencephalon. However, the presence of an *Irx3*- and *Tcf4*-free territory rostral to the posterior diencephalon at the 17-somite stage and their later anterior expansion argues that the increased cell death identified in the anterior brain territory of 17-somite stage embryos is responsible for the caudalized brain phenotype of *Six3*-null embryos. Nevertheless, it could also be argued that a possible change in cell fate could also contribute to this phenotype, or, that similar to what has been described in chicken embryos (Arnold-Aldea and Cepko, 1996; Golden and Cepko, 1996; Larsen et al., 2001), cell dispersion between the posterior and anterior diencephalon might have also contributed to caudalization at this stage.

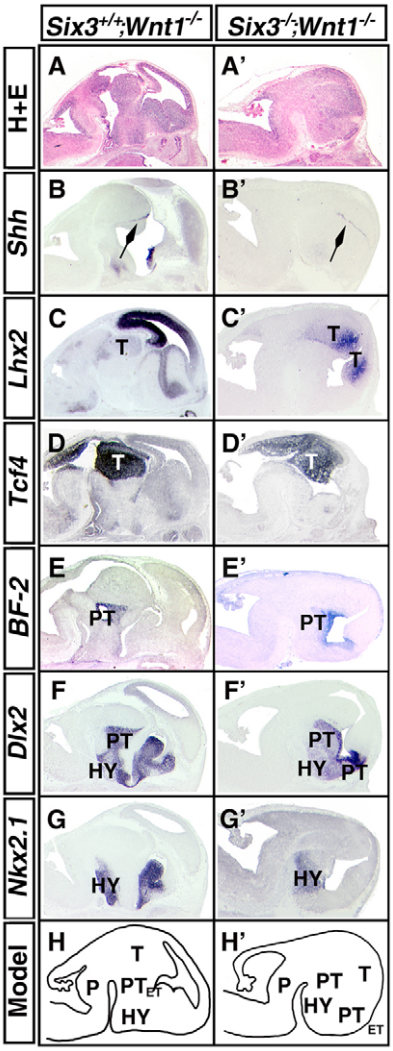

Fig. 6. ZLI is rescued in the *Six3;Wnt1* double-null brain. Hematoxylin and Eosin staining of sagittal sections from E14.5 *Wnt1*-null (*Six*$^{+/+}$;*Wnt1*$^{-/-}$, **A**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (**A′**) mouse brains. As indicated by *Shh* expression, the ZLI (arrow) is normally detected in the alar plate of *Wnt1*$^{-/-}$ (**B**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brains (**B′**). *Lhx2* and *Tcf4* expression is detected in the thalamus of *Wnt1*$^{-/-}$ (**C**,**D**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (**C′**,**D′**) brains. *BF-2* and *Dlx2* labeled the prethalamus, indicating the presence of these structures in the *Wnt1*$^{-/-}$ (**E**,**F**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (**E′**,**F′**) brains. As revealed by *Dlx2* and *Nkx2.1* analyses, the hypothalamus was also present in *Wnt1*$^{-/-}$ (F,**G**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (F′,**G′**) brains at E14.5. Models representing the *Six3*$^{+/+}$;*Wnt1*$^{-/-}$ (**H**) and *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ (**H′**) brains at this stage are included for clarity. The thalamus and prethalamus are rescued in *Six3*$^{-/-}$;*Wnt1*$^{-/-}$ brains (H′). Anterior is to the right. ET, eminentia thalami; HY, hypothalamus; P, Pons; PT, prethalamus; T, thalamus.

Six3 activity is not required for the formation or maintenance of the mammalian ZLI

The interaction between prospective thalamus and prethalamus specifies the position of the ZLI in chicken (Kobayashi et al., 2002; Braun et al., 2003; Echevarria et al., 2003; Vieira et al., 2005) and zebrafish (Scholpp et al., 2006) embryos. In chicken, it has been proposed that the abutting expression of *Six3* rostrally and *Irx3* caudally marks the position where the ZLI will form (Kobayashi et al., 2002). However, in mouse, the *Irx1* expression domain, a gene whose expression pattern is similar to that of chicken *Irx3* (Cohen et al., 2000; Hirata et al., 2006b), does not overlap with that of *Six3* (data not shown). Furthermore, the absence of *Six3* activity does not affect the establishment of the initial domain of *Shh* expression in the prospective ZLI. These data support the proposal that unlike in chicken, in mammals *Six3* does not directly affect ZLI localization; instead, it indirectly maintains the anterior character of the developing rostral brain.

In mammals, other genes expressed in the prospective prethalamus have a role in ZLI localization. *Fezf1* and *Fezf2* are expressed in this region and their deficit inhibits the formation of the prospective prethalamus and ZLI (Hirata et al., 2004; Hirata et al., 2006b). Here we showed that in *Six3*-null embryos the ZLI is not present at late developmental stages, and the expression domains of *Fezf1* and *Fezf2* are greatly reduced. *Fezf1* expression and the presence of the ZLI were recovered in the *Six3;Wnt1* double-null embryos. These results suggest that at the early stages of ZLI localization, *Six3* acts upstream of *Fezf1*/*Fezf2* and maintains the anterior character of the tissue rostral to the future ZLI by repressing dorsal Wnt signaling. By taking advantage of the available *Six3* conditional strain, we determined that *Shh* expression in the ZLI is independent of *Six3* activity; therefore, the establishment and maintenance of the ZLI in mammals do not require *Six3* activity.

Six3 is required for the formation of the telencephalon

The lack of telencephalon in *Six3*-null embryos may result from the ectopic anterior expansion of *Wnt1* (Lagutin et al., 2003). However, ectopic expression of *Wnt1* in the mouse ANE was not sufficient to affect the formation of the telencephalon and rostral diencephalon (Ligon et al., 2003). These results suggest that the ectopic anterior expansion of *Wnt1* expression in *Six3*-null embryos is not responsible for the brain truncation; instead, this aberration could be directly related to the lack of Six3 activity. This possibility was supported by our concurrent inactivation of *Six3* and *Wnt1* in mice, which showed that *Wnt1* removal is not sufficient to rescue telencephalic formation.

In mice, other Wnt family members such as *Wnt8b* and *Wnt3a* are expressed in the ANE and roof plate (Parr et al., 1993). *wnt8b* antagonizes telencephalon formation in zebrafish, and reduced Wnt signaling in *mbl*$^{-/-}$ zebrafish embryos by the abrogation of *wnt8b* activity restores the telencephalon (Houart et al., 2002). *Wnt3a*;*Wnt1* double-null mouse embryos exhibit a more-severe brain phenotype

than do the *Wnt1*$^{-/-}$ (McMahon and Bradley, 1990) or *Wnt3a*$^{-/-}$ (Lee et al., 2000) single-null brains (Ikeya et al., 1997; Megason and McMahon, 2002). We showed that *Wnt3a* expression was anteriorly expanded in the *Six3*-null brain. This result suggests that other Wnt family members (e.g. *Wnt3a*) could partially compensate for the early absence of *Wnt1* activity by allowing the formation of the diencephalon, but not of the telencephalon.

Using *Six3-Cre* or *CAGG-CreERT2* strains to remove *Six3* activity from the brain at E9.0-9.5 reduced the size of the telencephalon. At these stages, *Six3* is expressed only in the ventral forebrain (Oliver et al., 1995); thus, this result indicates that in addition to its early role in the ANE, *Six3* activity in the ventral forebrain is required during later stages of mammalian telencephalic development to facilitate the expansion/growth of this region. A similar role has also been suggested in zebrafish and frog embryos (Kobayashi et al., 1998; Ando et al., 2005; Gestri et al., 2005).

Rostral diencephalic development depends on Six3 repression of *Wnt1* posteriorizing signals

Conditional inactivation of *Six3* confirmed not only that Six3 activity does not maintain *Shh* expression in the ZLI, but also that *Six3* activity prior to E9.0 is necessary for rostral diencephalon development. Patterning of the forebrain into telencephalic, eye and diencephalic regions is the result of graded expression of Wnt signaling in the anterior neural plate (Houart et al., 2002; Wilson and Houart, 2004). Accordingly, we argue that anterior neural tissue is more susceptible to subtle changes in Wnt signaling.

In the *Six3*-null brain, early ectopic expansion of *Wnt1* expression is not sufficient to block rostral diencephalic formation. However, at around the 17-somite stage, the abnormal anterior expansion of Wnt activity causes the mutant brain to posteriorize. As a consequence, nearly all the prospective prethalamus is lost at the 22- to 24-somite stage and is later replaced by the pretectum. Because the prethalamus is not maintained, the ZLI and the boundary between thalamus and prethalamus will not form. Defects in ZLI formation or suppression of *Shh* expression in the ZLI promote caudalization of the mouse diencephalon (Hirata et al., 2006b) and defective thalamic and prethalamic induction in chicken (Kiecker and Lumsden, 2004; Vieira and Martinez, 2006) and zebrafish (Scholpp et al., 2006; Scholpp et al., 2007) embryos. Therefore, absence of the ZLI is probably at least partially responsible for the late-caudalization phenotype of the *Six3*-mutant brain.

The generation of *Six3;Wnt1* double-null mutants revealed that repression of *Wnt1* activity by Six3 in the early ANE is required to protect the rostral diencephalon from posteriorizing signals, to allow the expansion of the ZLI and to pattern the diencephalon.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/441/DC1

References

Ando, H., Kobayashi, M., Tsubokawa, T., Uyemura, K., Furuta, T. and Okamoto, H. (2005). Lhx2 mediates the activity of Six3 in zebrafish forebrain growth. *Dev. Biol.* **287**, 456-468.

Arnold-Aldea, S. A. and Cepko, C. L. (1996). Dispersion patterns of clonally related cells during development of the hypothalamus. *Dev. Biol.* **10**, 148-161.

Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M. and De Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68**, 45-57.

Bosse, A., Zulch, A., Becker, M. B., Torres, M., Gomez-Skarmeta, J. L., Modolell, J. and Gruss, P. (1997). Identification of the vertebrate Iroquois homeobox gene family with overlapping expression during early development of the nervous system. *Mech. Dev.* **69**, 169-181.

Braun, M. M., Etheridge, A., Bernard, A., Robertson, C. P. and Roelink, H. (2003). Wnt signaling is required at distinct stages of development for the induction of the posterior forebrain. *Development* **130**, 5579-5587.

Bulfone, A., Smiga, S. M., Shimamura, K., Peterson, A., Puelles, L. and Rubenstein, J. L. (1995). T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron* **15**, 63-78.

Carl, M., Loosli, F. and Wittbrodt, J. (2002). Six3 inactivation reveals its essential role for the formation and patterning of the vertebrate eye. *Development* **129**, 4057-4063.

Cho, E. A. and Dressler, G. R. (1998). TCF-4 binds beta-catenin and is expressed in distinct regions of the embryonic brain and limbs. *Mech. Dev.* **77**, 9-18.

Cohen, D. R., Cheng, C. W., Cheng, S. H. and Hui, C. C. (2000). Expression of two novel mouse Iroquois homeobox genes during neurogenesis. *Mech. Dev.* **91**, 317-321.

Del Bene, F., Tessmar-Raible, K. and Wittbrodt, J. (2004). Direct interaction of geminin and Six3 in eye development. *Nature* **427**, 745-749.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Echevarria. D., Vieira, C., Gimeno, L. and Martinez, S. (2003). Neuroepithelial secondary organizers and cell fate specification in the developing brain. *Brain Res. Brain Res. Rev.* **43**, 179-191.

Fujii, T., Pichel, J. G., Taira, M., Toyama, R., Dawid, I. B. and Westphal, H. (1994). Expression patterns of the murine LIM class homeobox gene lim1 in the developing brain and excretory system. *Dev. Dyn.* **199**, 73-83.

Furuta, Y., Lagutin, O., Hogan, B. L. and Oliver, G. C. (2000). Retina- and ventral forebrain-specific Cre recombinase activity in transgenic mice. *Genesis* **26**, 130-132.

Gamse, J. and Sive, H. (2000). Vertebrate anteroposterior patterning: the Xenopus neurectoderm as a paradigm. *BioEssays* **22**, 976-986.

Garel, S., Marin, F., Mattei, M. G., Vesque, C., Vicent, A. and Charnay, P. (1997). Family of Ebf/Olf-1-related genes potentially involved in neuronal differentiation and regional specification in the central nervous system. *Dev. Dyn.* **210**, 191-205.

Gestri, G., Carl, M., Appolloni, I., Wilson, S. W., Barsacchi, G. and Andreazzoli, M. (2005). Six3 functions in anterior neural plate specification by promoting cell proliferation and inhibiting Bmp4 expression. *Development* **132**, 2401-2413.

Golden, J. A. and Cepko, C. L. (1996). Clones in the chick diencephalon contain multiple cell types and siblings are widely dispersed. *Development.* **122**, 65-78.

Gradwohl, G., Fode, C. and Guillemot, F. (1996). Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. *Dev. Biol.* **180**, 227-241.

Hatini, V., Tao, W. and Lai, E. (1994). Expression of winged helix genes, BF-1 and BF-2, define adjacent domains within the developing forebrain and retina. *J. Neurobiol.* **25**, 1293-1309.

Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev. Biol.* **244**, 305-318.

Heisenberg, C. P., Houart, C., Take-Uchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R. et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434.

Hirata, T., Suda, Y., Nakao, K., Narimatsu, M., Hirano, T. and Hibi, M. (2004). Zinc finger gene fez-like functions in the formation of subplate neurons and thalamocortical axons. *Dev. Dyn.* **230**, 546-556.

Hirata, T., Nakazawa, M., Yoshihara, S., Miyachi, H., Kitamura, K., Yoshihara, Y and Hibi, M. (2006a). Zinc-finger gene Fez in the olfactory sensory neurons regulates development of the olfactory bulb non-cell-autonomously. *Development* **133**, 1433-1443.

Hirata, T., Nakazawa, M., Muraoka, O., Nakayama, R., Suda, Y. and Hibi, M. (2006b). Zinc-finger genes Fez and Fez-like function in the establishment of diencephalon subdivisions. *Development* **133**, 3993-4004.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M. and Wilson, S. (2002). Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. *Neuron* **35**, 255-265.

Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P. and Takada, S. (1997).

Wnt signaling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-970.
Jean, D., Bernier, G. and Gruss, P. (1999). Six6 (Optx2) is a novel murine Six3-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk. *Mech. Dev.* **84**, 31-40.
Jones, C. M., Lyons, K. M. and Hogan, B. L. (1991). Involvement of Bone Morphogenic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in mouse. *Development* **111**, 531-542.
Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signaling regulates anteroposterior neural patterning in Xenopus. *Development* **128**, 4189-4201.
Kiecker, C. and Lumsden, A. (2004). Hedgehog signaling from the ZLI regulates diencephalic regional identity. *Nat. Neurosci.* **7**, 1242-1249.
Kim, C. H., Oda, T., Itoh, M., Jiang, D., Artinger, K. B., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B. (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 913-916.
Kishigami, S., Komatsu, Y., Takeda, H., Nomura-Kitabayashi, A., Yamauchi, Y., Abe, K., Yamamura, K. and Mishina, Y. (2006). Optimized beta-galactosidase staining method for simultaneous detection of endogenous gene expression in early mouse embryos. *Genesis* **44**, 57-65.
Kobayashi, D., Kobayashi, M., Matsumoto, K., Ogura, T., Nakafuku, M. and Shimamura, K. (2002). Early subdivisions in the neural plate define distinct competence for inductive signals. *Development* **129**, 83-93.
Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-2982.
Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.
Lagutin, O. V., Zhu, C. C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H. R., McKinnon, P. J., Solnica-Krezel, L. and Oliver, G. (2003). Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. *Genes Dev.* **17**, 368-379.
Larsen, C. W., Zeltser, L. M. and Lumsden, A. (2001). Boundary formation and compartition in the avian diencephalon. *J. Neurosci.* **21**, 4699-4711.
Lavado, A. and Oliver, G. (2007). Prox1 expression patterns in the developing and adult murine brain. *Dev. Dyn.* **236**, 518-524.
Lee, S. M., Tole, S., Grove, E. and McMahon, A. P. (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. *Development* **127**, 457-467.
Lekven, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.
Ligon, K. L., Echelard, Y., Assimacopoulos, S., Danielian, P. S., Kaing, S., Grove, E. A., McMahon, A. P. and Rowitch, D. H. (2003). Loss of Emx2 function leads to ectopic expression of Wnt1 in the developing telencephalon and cortical dysplasia. *Development* **130**, 2275-2287.
Liu, W., Lagutin, O. V., Mende, M., Streit, A. and Oliver, G. (2006). Six3 activation of Pax6 expression is essential for mammalian lens induction and specification. *EMBO J.* **25**, 5383-5395.
Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.
McMahon, A. P. and Bradley, A. (1990). The Wnt-1 (int-1). proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.
McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.
McWhirter, J. R., Goulding, M., Weiner, J. A., Chun, J. and Murre, C. (1997). A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. *Development* **124**, 3221-3232.
Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-2098.
Miura, H., Yanazawa, M., Kato, K. and Kitamura, K. (1997). Expression of a novel aristaless related homeobox gene 'Arx' in the vertebrate telencephalon, diencephalon and floor plate. *Mech. Dev.* **65**, 99-109.
Nakagawa, Y. and O'Leary, D. D. (2001). Combinatorial expression patterns of LIM-homeodomain and other regulatory genes parcellate developing thalamus. *J. Neurosci.* **21**, 2711-2725.
Niehrs, C. (1999). Head in the WNT: the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.
Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* **121**, 4045-4055.
Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.
Pasquier, L., Dubourg, C., Gonzales, M., Lazaro, L., David, V., Odent, S. and Encha-Razavi, F. (2005). First occurrence of aprosencephaly/atelencephaly and holoprosencephaly in a family with a SIX3 gene mutation and phenotype/genotype correlation in our series of SIX3 mutations. *J. Med. Genet.* **42**, e4.
Porteus, M. H., Bulfone, A., Ciaranello, R. D. and Rubenstein, J. L. (1991). Isolation and characterization of a novel cDNA clone encoding a homeodomain that is developmentally regulated in the ventral forebrain. *Neuron* **7**, 221-229.
Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* **133**, 89-98.
Qiu, M., Shimamura, K., Sussel, L., Chen, S. and Rubenstein, J. L. (1998). Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. *Mech. Dev.* **72**, 77-88.
Rhinn, M., Lun, K., Luz, M., Werner, M. and Brand, M. (2005). Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by Wnt8 signaling. *Development* **132**, 1261-1272.
Ribeiro, L. A., El-Jaick, K. B., Muenke, M. and Richieri-Costa, A. (2006). SIX3 mutations with holoprosencephaly. *Am. J. Med. Genet. A* **140**, 2577-2583.
Rinkwitz-Brandt, S., Justus, M., Oldenettel, I., Arnold, H. H. and Bober, E. (1995). Distinct temporal expression of mouse Nkx-5.1 and Nkx-5.2 homeobox genes during brain and ear development. *Mech. Dev.* **52**, 371-381.
Sasaki, H. and Hogan, B. L. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.
Schaeren-Wiemers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.
Scholpp, S., Wolf, O., Brand, M. and Lumsden, A. (2006). Hedgehog signaling from the zona limitans intrathalamica orchestrates patterning of the zebrafish diencephalon. *Development* **133**, 855-864.
Scholpp, S., Foucher, I., Staudt, N., Peukert, D., Lumsden, A. and Houart, C. (2007). Otx1l, Otx2 and Irx1b establish and position the ZLI in the diencephalon. *Development* **134**, 3167-3176.
Sheng, H. Z., Bertuzzi, S., Chiang, C., Shawlot, W., Taira, M., Dawid, I. and Westphal, H. (1997). Expression of murine Lhx5 suggests a role in specifying the forebrain. *Dev. Dyn.* **208**, 266-277.
Shimamura, K. and Rubenstein, J. L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.
Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.
Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992a). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.
Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. and Boncinelli, E. (1992b). Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J.* **11**, 2541-2550.
Smith, S. M. and Cartwright, M. M. (1997). Spatial visualization of apoptosis using a whole-mount in situ DNA end-labeling technique. *Biotechniques* **22**, 832-834.
Stern, C. D. (2002). Induction and initial patterning of the nervous system – the chick embryo enters the scene. *Curr. Opin. Genet. Dev.* **12**, 447-451.
Stern, C. D. (2005). Neural induction: old problem, new findings, yet more questions. *Development* **132**, 2007-2021.
Tao, W. and Lai, E. (1992). Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron* **8**, 957-966.
Vieira, C. and Martinez, S. (2006). Sonic hedgehog from the basal plate and the zona limitans intrathalamica exhibits differential activity on diencephalic molecular regionalization and nuclear structure. *Neuroscience* **143**, 129-140.
Vieira, C., Garda, A. L., Shimamura, K. and Martinez, S. (2005). Thalamic development induced by Shh in the chick embryo. *Dev. Biol.* **284**, 351-363.
Wilson, S. W. and Rubenstein, J. L. (2000). Induction and dorsoventral patterning of the telencephalon. *Neuron* **28**, 641-651.
Wilson, S. W. and Houart, C. (2004). Early steps in the development of the forebrain. *Dev. Cell* **6**, 167-181.
Yamaguchi, T. P. (2001). Heads or Tails: Wnts and anterior-posterior patterning. *Curr. Biol.* **11**, 713-724.
Zeltser, L. M. (2005). Shh-dependent formation of the ZLI is opposed by signals from the dorsal diencephalon. *Development* **132**, 2023-2033.
Zhu, C. C., Dyer, M. A., Uchikawa, M., Kondoh, H., Lagutin, O. V. and Oliver, G. (2002). Six3-mediated auto repression and eye development requires its interaction with members of the Groucho-related family of co-repressors. *Development* **129**, 2835-2849.