Zinc-finger genes Fez and Fez-like function in the establishment of diencephalon subdivisions

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Fez and Fez-like (Fezl) are zinc-finger genes that encode transcriptional repressors expressed in overlapping domains of the forebrain. By generating Fez;Fezl-deficient mice we found that a redundant function of Fez and Fezl is required for the formation of diencephalon subdivisions. The caudal forebrain can be divided into three transverse subdivisions: prethalamus (also called ventral thalamus), thalamus (dorsal thalamus) and pretectum. Fez;Fezl-deficient mice showed a complete loss of prethalamus and a strong reduction of the thalamus at late gestation periods. Genetic marker analyses revealed that during early diencephalon patterning in Fez;Fezl-deficient mice, the rostral diencephalon (prospective prethalamus) did not form and the caudal diencephalon (prospective thalamus and pretectum) expanded rostrally. Fez;Fezl-deficient mice also displayed defects in the formation of the zona limitans intrathalamica (ZLI), which is located on the boundary between the prethalamus and thalamus. Fez and Fezl are expressed in the region rostral to the rostral limit of Irx1 expression, which marks the prospective position of the ZLI. Transgene-mediated misexpression of Fez1 or Fez caudal to the ZLI repressed the caudal diencephalon fate and affected the formation of the Shh-expressing ZLI. These data indicate that Fez and Fezl repress the caudal diencephalon fate in the rostral diencephalon, and ZLI formation probably depends on Fez/Fezl-mediated formation of diencephalon subdivisions.

KEY WORDS: Fez (Fezf1) Fez-like (Fezf2), Zinc finger, Forebrain, Telencephalon, Diencephalon, Zona limitans intrathalamica, Thalamus, Prethalamus, Pretectum, Prosomere, Mouse, Transcriptional repressor

INTRODUCTION

The forebrain of adult mammals is one of the most complicated biological structures; it is essential for higher neural functions, such as memory, emotion, reasoning and the planning of coordinated movements. Various models for forebrain subdivisions (neuromeres) have been developed over the past 10 years (reviewed by Kiecker and Lumsden, 2005). Among them, Puelles and Rubenstein and colleagues proposed a neuromeric organization of the forebrain on the basis of the differential expression of neural marker genes and morphological considerations (the so called ‘prosomeric model’) (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003; Rubenstein et al., 1994; Rubenstein et al., 1998). In the most recent model (Puelles and Rubenstein, 2003), the forebrain can be divided into rostral and caudal regions. The rostral part of the forebrain is the telencephalon and can be divided into several anatomical and functional territories, including the pallium, subpallium, preoptic area and hypothalamus. The caudal part of the forebrain is the diencephalon and can be divided into three transverse subdivisions known as prosomeres: the prethalamus (also called the ventral thalamus, p3), thalamus (also known as the dorsal thalamus, p2), and pretectum (p1). The eminentia thalamic, habenula/epithalamus and posterior commissures are located dorsally to the prethalamus, thalamus and pretectum, respectively.

Although the structures of the diencephalic subdivisions become obvious in mice late in gestation, individual subdivisions can be distinguished by their expression of genetic markers at the beginning of forebrain patterning. Members of the Dlx family of genes are expressed in the prethalamus, Gbx2 is expressed in the thalamus (Bullock et al., 1993) and Ebf1 and Lhx1 are expressed in the anterior and posterior pretectum, respectively (Barnes et al., 1994; Garel et al., 1997). Studies of genetically modified mice have revealed several genes involved in the patterning and/or development of the diencephalon. These include Pax6, Otx2, Emx1 and Six3 (Kimura et al., 2005; Kurokawa et al., 2004a; Kurokawa et al., 2004b; Lagutin et al., 2003; Stykova et al., 1996; Suda et al., 2001). However, it is largely unknown how these genes function in the formation of the diencephalon subdivisions and what other genes are involved in diencephalon patterning.

The zona limitans intrathalamica (ZLI) is located on the boundary between the prethalamus and thalamus (Larsen et al., 1995). The ZLI marks the interface between regions of different ability to respond to inductive signals such as Fgf8 and Shh; for instance, in neural tissue rostral to the ZLI, Fgf8 induces the expression of Foxg1 (also called Bf-1), but caudal to the ZLI it induces the expression of En2 (Shimamura and Rubenstein, 1997). The ZLI also has inductive influences on the adjacent subdivisions (prethalamus and thalamus). Shh expressed in the ZLI is involved in development of the prethalamus and thalamus in chick and zebrafish embryos; inhibition of Shh signaling represses the expression of the prethalamus and thalamus markers (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). In chick embryos, the future position of the ZLI is at the abutting expression domains of two homeobox genes, Six3 rostrally and Irx3 caudally (Kobayashi et al., 2002). Six3 and Irx3 regulate expression of each other, and the misexpression of Six3 or Irx3 affects the formation of the prethalamus and thalamus (Braun et al., 2003; Kobayashi et al., 2002). Irx3 expression confers...
competence to respond to the Fgf8 and Shh inductive signals by expressing thalamus-specific genes (Kiecker and Lumsden, 2004; Kobayashi et al., 2002). The expression of Six3 and Irx3 is regulated by Wnt signaling (Braun et al., 2003); Wnt1, Wnt3 and Wnt3a are expressed in the dorsal neural tissue caudal to the prospective ZLI when the expression of Six3 and Irx3 begins (Braun et al., 2003; Lagutin et al., 2003; Roelink and Nusse, 1991; Salinas and Nusse, 1992). These reports suggest that Wnt signaling controls the rostro-caudal polarity of the forebrain through the regulation of Six3 and Irx3, and the mutually repressive interaction of Six3 and Irx3 controls the position of the ZLI and thus confers differential competence to respond to the inductive signals to form the prethalamic region, and that ZLI formation depends on Foxg1 activity repressing the caudal diencephalon fate in the prospective forebrain. Here, we generated mice deficient in both the Fez and Fezl genes. The Fez/Fezl-deficient mice show no abnormalities in neural development in both zebrafish and mice. The zebrafish mutant too few (tfw) has a fezl gene mutation and displays a loss or reduction of dopaminergic neurons in the hypothalamus (Lewkowitz et al., 2003). Fez-deficient mice show no abnormalities in the dopaminergic neurons, but do show abnormal formation of the subplate neurons and thalamocortical axons, and loss of the fornix/fimbria system (Hirata et al., 2004). Fezl has also been shown to be required for the development of subcerebral projection neurons in layer V of the neocortex (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Fez-deficient mice display abnormal olfactory sensory neuronal projections and olfactory bulb formation (Hirata et al., 2006). The relatively weak forebrain phenotypes of Fez and Fezl-deficient mice suggest that Fez and Fezl function redundantly in the patterning and development of the forebrain.

Here, we generated mice deficient in both the Fez and Fezl genes. The Fez/Fezl-deficient mice showed defects in the rostro-caudal patterning of the diencephalon. We found that Fez and Fezl control the rostro-caudal patterning of the diencephalon by repressing the caudal diencephalon fate in the prospective prethalamic region, and that ZLI formation depends on Fez/Fezl-mediated formation of diencephalon subdivisions.

**MATERIALS AND METHODS**

**Mouse mutants and gene naming**

A mouse Fezl cDNA fragment (GenBank Accession Number A3129012) was originally reported as the mouse ortholog of Xenopus Fez (Matsuo-Takasaki et al., 2000). However, comparing the sequences of zebrafish fez (AB207804) and fezl (AB041824), mouse Fez homolog (AK014242), and the full-length clone of mouse Fezl (AB042399) (Hashimoto et al., 2000; Hirata et al., 2006) revealed that the mouse gene reported by Matsuo-Takasaki et al. is more similar to zebrafish fez1 than to Xenopus Fez (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000). Therefore, we refer to AB042399 as mouse Fez1 (Hashimoto et al., 2000; Hirata et al., 2004), and to AK014242, which is more similar to Xenopus Fez, as mouse Fezl (Hirata et al., 2006). Fez+/– and Fezl+/– mice were previously described (Hirata et al., 2006; Hirata et al., 2004). Both the Fez+/– and Fezl+/– heterozygous mice were originally established in a 129sv genetic background and backcrossed to the C57BL/6 background for several generations. Fez+/–Fezl+/– double heterozygous mice were generated by crossing Fez+/– and Fezl+/– heterozygotes, and were used to generate Fez−/−Fezl−/− embryos. Mouse was housed in an environmentally controlled room at the Animal Facility of the Center for Developmental Biology (CDB), RIKEN, under the guidelines for animal experiments from RIKEN CDB. The genotypes of newborn mice and embryos were determined by PCR analysis (Hirata et al., 2006; Hirata et al., 2004). The primer sequences and PCR conditions are available on request. Noon of the day on which the vaginal plug was detected was designated as embryonic day (E) 0.5.

**Histological sections and Nissl staining**

Brains or embryos were fixed with Carnoy’s solution at room temperature overnight. Specimens were dehydrated and embedded in paraffin. Serial sections were prepared and stained with 0.1% Cresyl Violet (MERCK).

**RNA probes and in situ hybridization**

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Specimens were gradually dehydrated in ethanol/H2O and stored in ethanol at –20°C. The protocol for in situ hybridization was described previously (Hirata et al., 2006; Hirata et al., 2004). Single-stranded digoxigenin-UTP-labeled (Roche) RNAs were used. In situ signals were detected with an anti-digoxigenin antibody and BM Purple (Roche). For two-color staining of the histological sections in Fig. 6D, Fez and Irx1 probes labeled with FITC-UTP and digoxigenin-UTP (Roche), were used respectively; BM Purple and Fast Red (Roche) were used for staining. For Fig. 6H-M, Fez and Fezl probes were labeled with FITC-UTP and Irx1 probe with digoxigenin-UTP. The hybridized signals for Fez and Fezl were detected by an alkaline phosphatase-conjugated anti-Fluorescein antibody (Roche). Histological sections and Nissl staining

**Generation and genotyping of transgenic mice**

We isolated an approximately 8.2 kbp enhancer/promoter region of Fez from the bacterial artificial chromosome clone containing the coding and non-coding regions of Fez (Hirata et al., 2004). To make the Fez enhancer/promoter-driven lacZ (β-galactosidase) transgene constructs, the 8.2 or 2.7 kbp enhancer/promoter region of Fez was connected to lacZ cDNA at the position of the translational initiation site of Fez (Fezl8.2p-lacZ, Fezl2.7p-lacZ). For expression of Fezl, its 2.7 kbp enhancer/promoter was connected to Fezl cDNA, followed by internal ribosome entry site (ires)-Gap43-Venus (Fezl7p-Fezl-ires-Gap43-Venus; a gift of Y. Yoshihara). Similarly, Otx2 FM enhancer (1.4 kbp) (Kurokawa et al., 2004a) and the promoter of mouse heart shock protein 68 (Mhsp68) (Sasaki and Hogan, 1996) were connected to Fezl cDNA and IRES-Gap43-Venus (OtxFM-
**RESULTS**

**Fez and Fezl function redundantly in forebrain formation**

To reveal whether Fez and Fezl have redundant roles in forebrain formation, we crossed Fez- and Fezl-deficient heterozygous mice (Hirata et al., 2006; Hirata et al., 2004) and generated mice deficient homozygously or heterozygously in the Fez and/or Fezl genes (Fig. 1). Fez+/Fezl-embryos showed a small olfactory bulb at E15.5 (Fig. 1F), as Fez- embryos do (Hirata et al., 2006). Fez+/Fezl- embryos showed defects in the formation of the dentate gyrus at E17.5 (Fig. 1K). Fez+/Fezl- embryos showed more severe

**Fig. 1. Morphology of the forebrain of wild-type, Fez+/Fezl+, Fez+/Fezl-, Fez-/Fezl+ and Fez-/Fezl- mice.** Coronal (A-D,I-L) and sagittal (E-H) sections of E15.5 (A-H), E17.5 (I-L) wild-type (A,E,I), Fez+/Fezl+ (B,F,J), Fez+/Fezl- (C,G,K), and Fez-/Fezl+ (D,H,L) mice. (M-Q) Sagittal sections of E18.5 Fez+/Fezl+ (M-O) and Fez-/Fezl+ (P,Q) mice at the level where posterior commissures (M,P, indicated by white arrowheads), habenulo-interpeduncular tracts (N,Q, white arrows) or mammillothalamic tracts (O, black arrowhead) were observed. Nissl staining. Fez+/Fezl+ mice had a very small olfactory bulb and Fez+/Fezl- showed a reduction of the dentate gyrus (arrow in K,L), compared with wild-type littermates. Fez-/Fezl+ mice showed loss of the olfactory bulb (arrow in H), dentate gyrus and the CA3 region of hippocampus (arrow in L), prethalamus, strong reduction of the thalamus (region between arrowheads) and a reduced neocortex. At E18.5, both Fez+/Fezl+ and Fez-/Fezl+ mice had a posterior commissure and habenulo-interpeduncular tract. The habenulo-interpeduncular tracts were small and abnormally located, due to severe reduction of the thalamus in Fez+/Fezl+ mice, Fez+/Fezl+ (O), but not Fez+/Fezl+ mice, had mammillothalamic tracts. DG, dentate gyrus; hit, habenulo-interpeduncular tract; hip, hippocampus; ht, hypothalamus; ma, mamillary region; mtt, mammillothalamic tracts; nct, neocortex; OB, olfactory bulb; pc, posterior commissure; th, thalamus; pt, pretectum; pth, prethalamus; s, septum; 3v, third ventricle. Scale bars: 0.5 mm.
phenotypes in the olfactory bulb and hippocampus: nearly complete loss of the olfactory bulb, stronger reduction of the CA3 region and loss of dentate gyrus in the hippocampus. Fez−/−Fezl−/− embryos also showed impaired neocortex formation. In addition, Fez−/−Fezl−/− embryos were found to have abnormalities in the formation of diencephalon, including loss of prethalamus and a strong reduction of the thalamus in size (Fig. 1H, arrowheads). These diencephalon defects were not observed in the Fez+/−Fezl+− and Fez+−Fezl+− embryos (Fig. 1F,G), indicating a redundant role of Fez and Fezl in the formation of diencephalon. Although it is intriguing to study the phenotypes of the olfactory bulb, hippocampus and neocortex in these mice, we restricted the focus of our study to the role of Fez and Fezl in diencephalon formation.

A series of sagittal sections revealed that the Fez−/−Fezl−/− embryos had posterior commissures and habenulo-interpeduncular tracts at E18.5 (Fig. 1P,Q), located on the dorsal side of the pretectum and on the boundary between the thalamus and pretectum, respectively (Fig. 1M,N). However, the habenulo-interpeduncular tracts in the Fez−/−Fezl−/− embryos were small and abnormally located (Fig. 1Q). This is probably due to the strong reduction of the thalamus. The Fez−/−Fezl−/− embryos did not have a mammillothalamic tract, which is located in between the prethalamus and thalamus (Fig. 1O). These data indicate that the Fez−/−Fezl−/− embryos had no prethalamus and displayed a strong reduction of thalamus, while having a relatively normal development of the pretectum.

**Loss of the prethalamus and expansion of the caudal diencephalon in Fez, Fezl double mutants**

We next examined the Fez;Fezl-deficient mice with various genetic markers at E12.5. Dlx1 is expressed in the ventral telencephalon (including the medial and lateral ganglionic eminences), hypothalamus and prethalamus, but not the thalamus (Fig. 2A).

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**Fig. 2. Defects in prethalamus and thalamus development and rostral expansion of the pretectum in Fez−/−Fezl−/− embryos at E12.5.**

(A-T) Expression of Dlx1 (marker for prethalamus and ganglionic eminence, A-D), Gbx2 (thalamus, E-H), Lhx1 (posterior pretectum and ZLI, I-L), Ebf1 (anterior pretectum, M-P) and Sox14 (rostral domain of thalamus, Q-T) in control, Fez−/−Fezl+−, Fez+−Fezl+− and Fez−/−Fezl−/− embryos was analyzed by in situ hybridization. Sagittal sections with anterior to the left. Dlx1 expression was not detected in the prethalamus but was maintained in the hypothalamus and ganglionic eminence in Fez−/−Fezl−/− embryos (D). Gbx2 expression was strongly reduced and detected in patches (arrows in H) in Fez−/−Fezl−/− embryos. Lhx1 expression was absent in the ZLI, but was not significantly affected in the posterior pretectum of Fez−/−Fezl−/− embryos (I-L). The expression domain of Ebf1 was expanded rostrally in Fez−/−Fezl−/− embryos (P), compared with that in the wild-type, Fez+−Fezl+− and Fez+−Fezl−/− littermates (M-O). The expression of Sox14 was abolished in Fez−/−Fezl−/− embryos (T). apt, anterior pretectum; GE, ganglionic eminence; ht, hypothalamus; ppt, posterior pretectum; pth, prethalamus; th, thalamus.
In the *Fez−/−Fezl−/−* embryos, *Dlx1* expression was maintained in the ganglionic eminences and hypothalamus, but was completely absent in the diencephalon (Fig. 2D). *Gbx2* is expressed strongly in the thalamus and weakly in the ganglionic eminences (Fig. 2E) (Bulfone et al., 1993). In *Fez−/−Fezl−/−* embryos, *Gbx2* expression in the thalamus was markedly reduced and shifted rostrally, but not abrogated (Fig. 2H). *Lhx1* is expressed in the posterior pretectum and ZLI (Fig. 2I) (Barnes et al., 1994; Fujii et al., 1994; Mastick et al., 1997; Suda et al., 2001). Although its expression in the posterior pretectum was not affected significantly, we found that it was not expressed in the ZLI in the *Fez−/−Fezl−/−* embryos (Fig. 2L). *Ebf1* is expressed in the anterior pretectum (Garel et al., 1997; Suda et al., 2001) (Fig. 2M), and its expression was expanded rostrally in the *Fez−/−Fezl−/−* embryos (Fig. 2P). *Sox14* is expressed in the rostral part of the thalamus (Hashimoto-Torii et al., 2003) (Fig. 2Q), and its expression was absent in the *Fez−/−Fezl−/−* embryos (Fig. 2T). Neither the *Fez−/−Fezl−/−* nor the *Fez+/−Fezl−/−* embryos showed abnormal expression of *Dlx1*, *Gbx2*, *Lim1*, *Ebf1* or *Sox14* at E12.5 (Fig. 2B,C,F,G,J,K,N,O,R,S), further confirming the strictly redundant function of *Fez* and *Fezl* in the diencephalon patterning. The data indicate that, in the *Fez−/−Fezl−/−* embryos at E12.5, the prethalamus did not form, the thalamus formed at a reduced size and the anterior pretectum expanded rostrally, suggesting that *Fez* and *Fezl* play an important role in rostro-caudal patterning of the diencephalon. Although we detected *Tuj-1*-positive postmitotic neurons in the thalamus at E11.5 in the *Fez−/−Fezl−/−* embryos (data not shown), the *Fez−/−Fezl−/−* embryos failed to form the rostral part of the thalamus.

**Fig. 3. Defects in telencephalon formation and regionalization of the diencephalon in *Fez−/−Fezl−/−* embryos.** Expression of *Emx2 (A-D), Pax6 (E-H), Dlx1 (I-L), Shh (M-P), Tcf4 (Q-T) and Lhx5 (U-X) in control (A,E,I,M,Q,U), *Fez−/−Fezl−/−* (B,F,J,N,R,V), *Fez+/−Fezl−/−* (C,G,K,O,S,W) and *Fez−/−Fezl−/−* embryos (D,H,L,P,T,X) at E10.5. (A-T) Whole-mount in situ hybridization and lateral views of anterior neuroectoderm, with anterior to the left. (U-X) Coronary sections of the diencephalon. *Emx2* expression in the dorsal telencephalon was reduced in *Fez−/−Fezl−/−* embryos. Pax6 expression was downregulated in the thalamus at this stage (marked by dots, E,F,G) in control, *Fez−/−Fezl−/−* and *Fez+/−Fezl−/−* embryos, but not in *Fez−/−Fezl−/−* embryos (arrow, H). The expression domain of Pax6 in the dorsal telencephalon was reduced but not significantly affected in the diencephalon except for the thalamus (region between arrowheads) in *Fez−/−Fezl−/−* embryos. *Dlx1* expression was detected in the ganglionic eminence (arrow, I,L) but not in the prethalamus (arrowhead, I,L) in *Fez−/−Fezl−/−* embryos. *Shh* expression was detected in the AEP, but not in the ZLI in *Fez−/−Fezl−/−* embryos (arrow, P) compared with controls (M-O). The domain expressing *Tcf4* at a high level (thalamus and pretectum region, marked by dots) was expanded in *Fez−/−Fezl−/−* embryos (T), compared with controls (Q-S). *Lhx5* expression was abolished in the prethalamus of *Fez−/−Fezl−/−* embryos (X). ht, hypothalamus; pth, prethalamus; th, thalamus.
(Sox14-positive region), the formation of which is known to depend on Shh from the ZLI (Hashimoto-Torii et al., 2003). The data indicate that the redundant function of Fez and Fezl also controls the rostro-caudal patterning of the thalamus directly or indirectly.

Defects in regionalization of the diencephalon in the Fez, Fezl double mutant

We analyzed the Fez;Fezl-deficient mice with genetic markers at E10.5, when forebrain patterning becomes apparent. Emx2 is expressed in the dorsal telencephalon (pallium) (Fig. 3A) (Yoshida et al., 1997), and its expression domain was reduced in the Fez–/–Fezl–/–, but not in the Fez+/–Fezl–/– or Fez+/–Fezl+/– embryos (Fig. 3B-D). This is consistent with the reduction of the neocortex and loss of the hippocampus, as Emx2 (and Emx1) is required for neocortical and hippocampal formation (Pellegrini et al., 1996; Yoshida et al., 1997). Pax6 is initially expressed in the dorsal telencephalon and diencephalon, but its expression in the thalamus is reduced by E10.5 (Fig. 3E) (Mastick et al., 1997; Stoykova and Gruss, 1994; Warren and Price, 1997). The rostral and caudal limits of Pax6 expression in the diencephalon were the same in Fez+/–Fezl–/– embryos as in wild type, Fez–/–Fezl–/– and Fez+/–Fezl+/– embryos (Fig. 3E-H). However, the Pax6 expression in the thalamus was not reduced in the Fez–/–Fezl–/– embryos (Fig. 3H), suggesting that the rostro-caudal patterning, but not the formation of the diencephalon, was affected in the Fez–/–Fezl–/– embryos. The Dlx1 expression in the prethalamus was already absent at E10.5 in Fez–/–Fezl–/– embryos (Fig. 3L), indicating that the prethalamus was not established. Sihh is expressed in the anterior entopeduncular area (AEP) of the ventral telencephalon, the basal plate of the entire neuroectoderm (including the hypothalamus) and the ZLI (Fig. 3M) (Echelard et al., 1993; Ericson et al., 1995) in the wild-type, Fez–/–Fezl–/– and Fez+/–Fezl+/– embryos, but its expression in the ZLI was absent in the Fez–/–Fezl–/– embryos (Fig. 3M-P), suggesting that the ZLI was not established in the absence of Fez and Fezl. Tcf4 is expressed at a high level in the thalamus and preetectum (Cho and Dressler, 1998; Korinek et al., 1998) in the wild type, Fez–/–Fezl–/– and Fez+/–Fezl+/– embryos, whereas the Tcf4-high domain expanded rostrally in the Fez–/–Fezl–/– embryos (Fig. 3Q-T). Lhx5 is expressed in the prethalamus in the wild type, Fez–/–Fezl–/– and Fez+/–Fezl+/– embryos (Fig. 3U-W) (Nakagawa and O’Leary, 2001), but was not detected in the Fez–/–Fezl–/– embryos (Fig. 3X). Taking these data together, loss of the prethalamus and expansion of the caudal diencephalon took place before E10.5, and the ZLI, the prethalamus-thalamus boundary, was not established in the absence of Fez and Fezl.

Fez and Fezl are involved in early rostro-caudal forebrain patterning

Formation and patterning of the forebrain are regulated by transcription factors and inductive signals expressed in specific rostro-caudal positions in the neuroectoderm at the beginning of neurogenesis and neural patterning. We examined gene expression in wild type and Fez–/–Fezl–/– embryos at around E9.5. The expression domain of Emx2 and Pax6 in the dorsal telencephalon was already reduced in the Fez–/–Fezl–/– embryos at E9.5 (Fig. 4A-D). Fgf8 was expressed in the commissural plate, the infundibulum of the hypothalamus, the dorsal part of the prethalamus (eminencia thalami) and the mid-hindbrain boundary (isthmus) in wild-type embryos (Fig. 4E) (Crossley and Martin, 1995), whereas its expression in the prospective prethalamus was specifically absent in the Fez–/–Fezl–/– embryos (Fig. 4F). Wnt3a is expressed in the dorsal neural tissue caudal to the prospective ZLI (Fig. 4G) (Roelink and Nusse, 1991; Salinas and Nusse, 1992), and the rostral limit of its expression was shifted rostrally in the Fez–/–Fezl–/– embryos (Fig. 4H). En2 expression in the midbrain was not affected in the Fez–/–Fezl–/– embryos (Fig. 4I). All these data indicate that the diencephalon patterning was already affected...
had shifted rostrally at E9.0, suggesting that *Fez* and *Fezl* are required to repress the *Irx1* expression in the rostral diencephalon. We found that the expression of *Six3* was not affected at E8.5 and 9.5 (Fig. 5J, data not shown for E8.5), suggesting that the expansion of the caudal diencephalon in the *Fez+Fezl+* embryos was independent of *Six3*.

**Complementary expression of Fez/Fezl and Irx1**

The expression of *Fez* and *Fezl* in the forebrain is initiated at E8.0 and 8.5, respectively (Hirata et al., 2006; Hirata et al., 2004) (Fig. 6B). At E12.5, *Fez* and *Fezl* are expressed in the pallium, septum, hypothalamus and prethalamus, which are located rostral to the ZLI (Hirata et al., 2004; Hirata et al., 2006). We examined the early expression domains of *Fez* and *Fezl*, and compared them with those of *Irx1*. At E8.5, the expression domain of *Fez* was slightly wider than that of *Fezl* (Fig. 6A,B), and the caudal limit of *Fez* abutted the rostral limit of *Irx1* (Fig. 6D). Two-color staining for *Fez* or *Fezl* with *Irx1* revealed that the expression of *Fez* and *Fezl* was strictly rostral to that of *Irx1* at E9.5 (Fig. 6H-M).

**Fez and Fezl repress the caudal diencephalon fate**

The expression of *Fez* and *Fezl* suggests that *Fez* and *Fezl* function in the region rostral to the ZLI and repress the caudal diencephalon fate. To address this issue, we misexpressed *Fez* or *Fezl* caudal to the ZLI using the *Fez* gene enhancer/promoter or the *Otx2* forebrain-midbrain (FM) enhancer. We constructed β-galactosidase (*lacZ*) reporter genes in which the *lacZ* gene was connected to the 8.2 kbp or 2.7 kbp enhancer/promoter region upstream of the translational initiation site of the mouse *Fez* gene (Fig. 7A), and examined the *lacZ* expression in the resulting transgenics (Fig. 7B-D). The 8.2 kbp *Fez* enhancer/promoter (*Fez2.7p*) drove the *lacZ* expression in a wider region at E8.5 than the 8.2 kbp promoter did (Fig. 7C). At E9.5 in transgenic mice with the 2.7 kbp *Fezl* enhancer/promoter (*Fezl2.7p*) drove the *lacZ* expression in a wider region at E8.5 than the 2.2 kbp promoter did (Fig. 7C). When neural patterning began. In chick, ZLI is positioned on the boundary of the expression domains of *Six3* and *Irx3*, and *Six3* and *Irx3* can repress the expression of each other, possibly determining the position of the ZLI (Kobayashi et al., 2002). In mouse, *Irx1* and *Irx3* display a similar expression profile in the neuroectoderm, but the rostral limit of the *Irx1* expression is more rostral than that of *Irx3* and is positioned on the ZLI (Bosse et al., 1997; Cohen et al., 2000). We examined the expression of *Irx1* in wild-type and double-mutant embryos (Fig. 5). The rostral limit of *Irx1* expression was independent of *Six3*. **Development**

![Figure 5](image_url)
suppressed Irx1 expression without affecting Foxg1 expression (Fig. 8W). These data indicate that Fez and Fezl can suppress the caudal diencephalon fate.

DISCUSSION
Role of Fez and Fezl in diencephalon patterning
The Fez+/−Fezl+/− mutant embryos, but not the Fez+/−Fezl−/− or Fez−/−Fezl−/− embryos, showed defects in patterning of the diencephalon (Fig. 1), indicating a strictly redundant role for Fez and Fezl in diencephalon development. This is consistent with the overlapping expression of Fez and Fezl in the prethalamus at later stages (Hirata et al., 2006; Hirata et al., 2004). Fez and Fezl, respectively, begin expression at E8 and 8.5 (Hirata et al., 2006; Hirata et al., 2004). We detected a defect in the rostro-caudal polarity of the diencephalon: rostral expansion of the Irx1 expression at E9.0, indicating that the Fez/Fezl-mediated diencephalon patterning starts soon after the onset of Fez and Fezl expression. Marker analyses showed that the prethalamic region was not established; instead, the caudal diencephalon, which includes the thalamus and the anterior pretectum, expanded rostrally in the double-mutant embryos (Figs 3, 4). There are two possible explanations for the phenotype of Fez;Fezl-deficient embryos: (1) transformation of the prethalamus into the thalamus; and (2) truncation of the prethalamus and the rostral shift of the caudal neural tissue. We found that the size of the diencephalon was not significantly different in the double-mutant embryos, compared with the wild-type embryos, when the rostro-caudal patterning in the diencephalon became abnormal (Figs 3, 4). Our data strongly suggest that the transformation of the prethalamus into the caudal diencephalon takes place during early neural patterning in the absence of Fez and Fezl (Fig. 9). Future studies including the Cre-loxP-mediated cell-fate mapping of Fez and Fezl-expressing cells will definitely clarify this issue. The misexpression of Fez or Fezl suppressed the caudal diencephalon fate and induced the expression of Dlx1, which normally marks the prethalamus, in the region caudal to the ZLI (Fig. 8). All of these data indicate that Fez and Fezl function to repress the caudal diencephalon fate and establish the prethalamus fate (Fig. 9). Although the caudal diencephalon was initially expanded, the thalamus eventually became smaller and the rostral part of the thalamus was missing in the Fez;Fezl-deficient embryos at E12.5 (Figs 1, 2). The later development of the thalamus is known to be dependent on inductive signals from the ZLI (discussed below). The loss of the ZLI in the Fez;Fezl-deficient embryos secondarily affects the development of the thalamus in these embryos (Fig. 9).

Both FEZ and FEZL contain an Eh1 repressor motif, which interacts with the Groucho/TLE family of transcriptional corepressors (Bae et al., 2003; Kobayashi et al., 2001; Muhr et al., 2001; Shimizu et al., 2002). The Eh1 repressor motif of zebrafish Fezl is required for at least part of the Fez function in this animal (Levkowitz et al., 2003), suggesting that FEZ and FEZL function as transcriptional repressors to regulate patterning of the diencephalon.
In the Fez:Fezl double-mutant embryos, expression domains of the caudal diencephalic genes, such as Irx1, Wnt3a and Tcf4 (high-expression domain), expanded rostrally, and misexpression of Fez or Fezl caudal to the ZLI inhibited the expression of Irx1 and Tcf4 in the caudal diencephalon (Figs 4, 5, 8). Fez and Fezl are expressed in the region rostral to the rostral limit of Irx1 expression, which marks the prospective position of the ZLI. Taking these findings together, we conclude that Fez and Fezl directly or indirectly repress the caudal diencephalon genes in the rostral diencephalon (Fig. 9). Identification of target genes for Fez and Fezl and/or chromatin immunoprecipitation assay of FEZ/FEZL-binding genomic fragments will help to clarify the precise mechanism by which Fez and Fezl control the rostro-caudal polarity of diencephalon.

SIX3 negatively regulates the caudally expressed Wnt1 (Lagutin et al., 2003) and functions as a transcriptional repressor (Kobayashi et al., 2001; Lopez-Rios et al., 2003; Zhu et al., 2002). Six3-deficient mice show strong reduction of the neural tissue rostral to the ZLI (Lagutin et al., 2003). Six3 negatively controls the expression of Irx3 in chick (Kobayashi et al., 2002). These reports suggest that the role of Fez and Fezl in diencephalon patterning is similar to that of Six3, at least in part. However, Six3-deficient mice have rudimentary tissue rostral to the ZLI, and express Shh in the ZLI (Lagutin et al., 2003), and Fez:Fezl-double mutants display complete loss of the prethalamus and ZLI, implying there is a difference between the functions of Six3 and Fez/Fezl. We examined the expression of Six3 in the Fez–/–Fezl–/– embryos, but did not observe any significant alteration in the Six3 expression at E8.5 and 9.5 (Fig. 5, data not shown for E8.5), suggesting that Six3 does not function downstream of Fez and Fezl. Rather, Six3 may function upstream of, or in parallel with, Fez and Fezl. Future studies examining Fez and Fezl expression in Six3-deficient embryos and combinatorial gene disruption of Six3 and Fez and/or Fezl will clarify this issue.

Fez and Fezl expression is regulated remains unclear. There are several genes with expression domains that overlap with those of Fez and Fezl. They include Pax6, Enx1/2, Dlx1/2/5/6 and Otx1/2 in addition to Six3 (Bulfone et al., 1993; Oliver et al., 1995; Simeone et al., 1992a; Simeone et al., 1993; Simeone et al., 1992b; Stuhmer et al., 2002; Walther and Gruss, 1991). These genes might be involved in the regulation of Fez and Fezl expression. The expression of zebrafish fezl and Xenopus Fez is negatively regulated by Wnt signaling (Hashimoto et al., 2000) (M. Matsuo-Takasaki, personal communication). Thus, the initial expression of Fez and Fezl may be controlled by the rostro-caudal polarity information, in which Wnt signaling is strongly involved (Niehrs, 2004). In this context, Fez and Fezl may link the rostro-caudal polarity information to the subdivision formation in the diencephalon.

Role of Fez and Fezl in formation of the ZLI

The ZLI position is predicted as abutting the expression domains of rostral Six3 and caudal Irx3 in chick embryos (Kobayashi et al., 2002). We found that it is predicted by the expression of rostrally expressed Fez and Fezl and caudally expressed Irx1 in mouse embryos (Fig. 6). Furthermore, the deficiency of both Fez and Fezl led to loss of the ZLI, and misexpression of Fez or Fezl could inhibit ZLI formation or shift its position (Figs 3, 8). These data indicate that Fez and Fezl are involved in the formation and position of the ZLI. The ZLI initially forms as a wedge-shaped structure on the boundary between the prethalamus and thalamus, which is characterized by a gap in Lfrg expression; subsequently, it collapses to a narrow band (Zeltser et al., 2001). It is not clear whether Fez and Fezl are expressed in the prospective ZLI domain, although they are not expressed in the ZLI at E12.5 (Hirata et al., 2006; Hirata et al., 2004). Fez and Fezl are expressed in the prethalamus, but they do not induce Shh expression there. Thus, Fez and Fezl are not
Fig. 8. Misexpression of Fez or Fezl affects rostro-caudal polarity in the diencephalon. Misexpression of Fez (A-Q) or Fezl (R-U) by the 2.7 kbp Fez enhancer/promoter (Fezl2.7p-Fezl-IRES-Venus, Fezl2.7p-Fez-IRES-Venus), or Fez by the FM enhancer of the Otx2 gene and mouse Hsp68 promoter (Otx2FM-Hsp68-Fez-IRES-Venus, V, W) affected the diencephalon subdivisions. Exogenous Fez expression was monitored by the expression of Venus attached to an IRES (Fig. 7) (fluorescence images, A,D,G,J,L,O,R,T,V). E9.5 embryos were analyzed by whole-mount in situ hybridization with Foxg1 and Irx1 (B,C,S,W). E10.5 embryos were analyzed by in situ hybridization with Tcf4 (E,F,U) or Shh (H,I,K). Sagittal sections of E12.5 embryos were analyzed with Dlx1 or Gbx2 probes (M,N,P,Q). (C,F,J,N,Q) Control non-transgenic embryos. Expression of Foxg1 in the telencephalon was not affected (caudal limit marked by arrows), but the rostral limit of Irx1 expression (marked by arrowheads) was shifted caudally in Fezl2.7p-Fezl-IRES-Venus (B), Fezl2.7p-Fez-IRES-Venus (S) and Otx2FM-Hsp68-Fez-IRES-Venus embryos (W), compared with the control (C). Tcf4-high expression domain in the thalamus and prethalamus was strongly reduced in the Fezl2.7p-Fezl-IRES-Venus (E) and Fezl2.7p-Fez-IRES-Venus (U) embryos. Shh expression in the ZLI (arrows) and ventral diencephalon was reduced (H, n=2/4), or Shh expression in the ZLI was shifted caudally (K, n=2/4) in the Fezl2.7p-Fezl-IRES-Venus embryos, compared with control (I). Dlx1 expression in the prethalamus was expanded caudally when the exogenous Fez-IRES-Venus was strongly expressed (M, n=1/2). Gbx2 expression in the thalamus was strongly reduced in the Fezl2.7p-Fezl-IRES-Venus embryos (P, n=2/3).
likely to be instructive factors, but rather to function as permissive factors. Alternatively, Fez and Fezl may regulate ZLI formation indirectly and non-cell-autonomously by controlling formation of the prethalamus. Grafting experiments in chick embryos show that Shh expression is induced in the interface between the prechordal (rostral to ZLI) and epichordal plate neuroepithelia (caudal to ZLI) (Vieira et al., 2005), suggesting that an interaction between prethalamus and thalamus is involved in the induction of ZLI. The complete loss of the prethalamus in the Fez+/Fezl+/embryos might lead to the loss of non-cell-autonomous signals (secreted or membrane-associated molecules) from the prethalamus and subsequently result in loss of the ZLI. In this scenario, formation of the ZLI may be controlled by signals from both the prethalamus and caudal diencephalon. Zli formation is also dependent on Shh emanating from the basal plate (Zeltser, 2005). Thus, Fez and Fezl cooperate with Shh from the basal plate to determine the position of the ZLI.

Inductive signals from the ZLI are required for the formation of the prethalamus and thalamus (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). Shh expressed in the ZLI is involved in the formation and patterning of the thalamus (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira et al., 2005). In the Fez+/Fezl+ embryos, the Shh-expressing ZLI was not established, and the thalamus became small, although the caudal diencephalon initially expanded rostrally. Therefore, the reduced thalamus is likely to be a secondary consequence of the loss of the ZLI in the Fez+/Fezl+ embryos (Fig. 9). Consistent with this, the Fez+/Fezl+ embryos showed complete loss of the Sox14 expression (Fig. 2), which is dependent on Shh from the ZLI (Hashimoto-Torii et al., 2003). Our findings support the inductive role of the ZLI in thalamus development.

In summary, Fez and Fezl are essential factors for development of the forebrain, playing an important role in rostro-caudal patterning of the diencephalon and in ZLI formation. The involvement of repressor-type zinc-finger proteins in forebrain formation provides a new mechanism for the formation and patterning of the forebrain subdivisions.

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References
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