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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 *Fezl* 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.

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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 (Bulfone 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*, *Emx2* and *Six3* (Kimura et al., 2005; Kurokawa et al., 2004a; Kurokawa et al., 2004b; Lagutin et al., 2003; Stoykova 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., 2001; Shimamura 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 prethalmus 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 prethalamus and thalamus. This hypothesis is based mainly on the results of misexpression studies and explant assays in the chicken embryo, but there is little genetic evidence to support it. *Six3*-deficient mice display a strong reduction of the neural tissue rostral to the ZLI, but still express *Shh* in the ZLI and rudimentary rostral tissue (Lagutin et al., 2003), suggesting that other genes may cooperate with or function parallel to *Six3* to determine the position of the ZLI or control the formation of the rostral diencephalon.

Fez (*Fezf1* – Mouse Genome Informatics) and *Fez-like* (*Fezl*, *Fezf2* – Mouse Genome Informatics) are closely related genes that encode transcriptional repressors containing six C2H2-type zinc fingers and an Eh1 (Engrailed homology 1) repressor motif, and were originally isolated as anterior neuroectoderm-specific genes in *Xenopus* and zebrafish (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000). Orthologs of these two genes exist in the puffer fish, zebrafish, mouse and human (Hirata et al., 2006). In mouse and zebrafish, *Fez* and *Fezl* are expressed in overlapping domains in the forebrain during development (Hashimoto et al., 2000; Hirata et al., 2006; Hirata et al., 2004; Matsuo-Takasaki et al., 2000). A number of studies have previously investigated the specific roles of *Fez* and *Fezl* in neural development in both zebrafish and mice. The zebrafish mutant *too few* (*tfu*) has a *fezl* gene mutation and displays a loss or reduction of dopaminergic neurons in the hypothalamus (Levkowitz et al., 2003). *Fezl*-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* redundantly 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 AI325906) 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 *fezl* than to *Xenopus Fez* (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000). Therefore, we refer to AB042399 as mouse *Fezl* (Hashimoto et al., 2000; Hirata et al., 2004), and to AK014242, which is more similar to *Xenopus Fez*, as mouse *Fez* (Hirata et al., 2006). $Fezl^{+/-}$ and $Fez^{+/-}$ 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 $Fez^{-/-}Fezl^{+/-}$, $Fez^{+/-}Fezl^{-/-}$ and $Fez^{-/-}Fezl^{-/-}$ embryos. Mice were 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 designed 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/H_2O 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) and BM Purple, and those for *Irx1* were detected by a peroxidase-conjugated anti-digoxigenin antibody (Roche) and tyramide signal amplification system (TSA-Plus Fluorescein System, PerkinElmer). The precise protocols for in situ hybridization are available on request. The probes were: *Dlx1* (Bulfone et al., 1993), *Gbx2* (Bulfone et al., 1993), *Lhx1* (Fujii et al., 1994), *Ebf1* (Garel et al., 1997), *Sox14* (Hashimoto-Torii et al., 2003), *Emx2* (Yoshida et al., 1997), *Pax6* (Walther and Gruss, 1991), *Shh* (Echelard et al., 1993), *Tcf4* (Ishibashi and McMahon, 2002), *Lhx5* (Nakagawa and O'Leary, 2001), *Fgf8* (Crossley and Martin, 1995), *Irx1* (a gift of T. Ogura), *Wnt3a* (Takada et al., 1994), *En2* (Joyner and Martin, 1987), *Foxg1* (Tao and Lai, 1992), *Fez* (Hirata et al., 2006) and *Fezl* (Hirata et al., 2004). In situ hybridization images were taken using an AxioPlan2 microscope or a SteREO Lumar V12, equipped with an AxioCam CCD camera (Zeiss). Figures were assembled using AxioVision version 4.3 and Adobe Photoshop CS2.

Generation and genotyping of transgenic mice

We isolated an approximately 8.2 kbp enhancer/promoter region of *Fezl* from the bacterial artificial chromosome clone containing the coding and non-coding regions of *Fezl* (Hirata et al., 2004). To make the *Fezl* enhancer/promoter-driven *lacZ* (β-galactosidase) transgene constructs, the 8.2 or 2.7 kbp enhancer/promoter region of *Fezl* was connected to *lacZ* cDNA at the position of the translational initiation site of *Fezl* (*Fezl8.2p-lacZ*, *Fezl2.7p-lacZ*). For misexpression of *Fezl*, its 2.7 kbp enhancer/promoter was connected to *Fezl* or *Fez* cDNA, followed by internal ribosomal entry site (IRES)-Gap43-Venus (*Fezl2.7p-Fezl-IRES-Venus* and *Fezl2.7p-Fez-IRES-Venus*) (IRES-Gap43-Venus, a gift of Y. Yoshihara). Similarly, *Otx2* FM enhancer (1.4 kbp) (Kurokawa et al., 2004a) and the promoter of mouse heat shock protein 68 (pHsp68) (Sasaki and Hogan, 1996) were connected to *Fezl* cDNA and IRES-Gap43-Venus (*OtxFM-*

Hsp68-FeZLIRES-Venus). The transgene-derived *Fezl* expression was monitored as Venus expression with an epifluorescence microscope AxioPlan2. Genotypes of the transgenic mice were analyzed by PCR with 5′-AAACCCTGGCGTTACCCAACT-3′ and 5′-ACGACAGTATCGGCCTCAGGA-3′ for the *lacZ* reporter lines, 5′-TGTGTCTGCAGAGAGTGCTGGCCTG-3′ and 5′-CTGGCTGCTGCTCACCCCAAGCTTT-3′ for the *Fezl2.7p-Fezl-IRES-Venus* and *OtxFM-Hsp68-Fezl-IRES-Venus* lines and 5′-AAAACGTATTTAGCCGAAAGGAAT-3′ and 5′-ACTTTACACACGAAGGGTCTGG-3′ for the *Fezl2.7p-Fez-IRES-Venus* lines. Transient transgenic embryos were generated and β-galactosidase staining was performed as described previously (Kimura et al., 1997; Kimura et al., 2000).

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

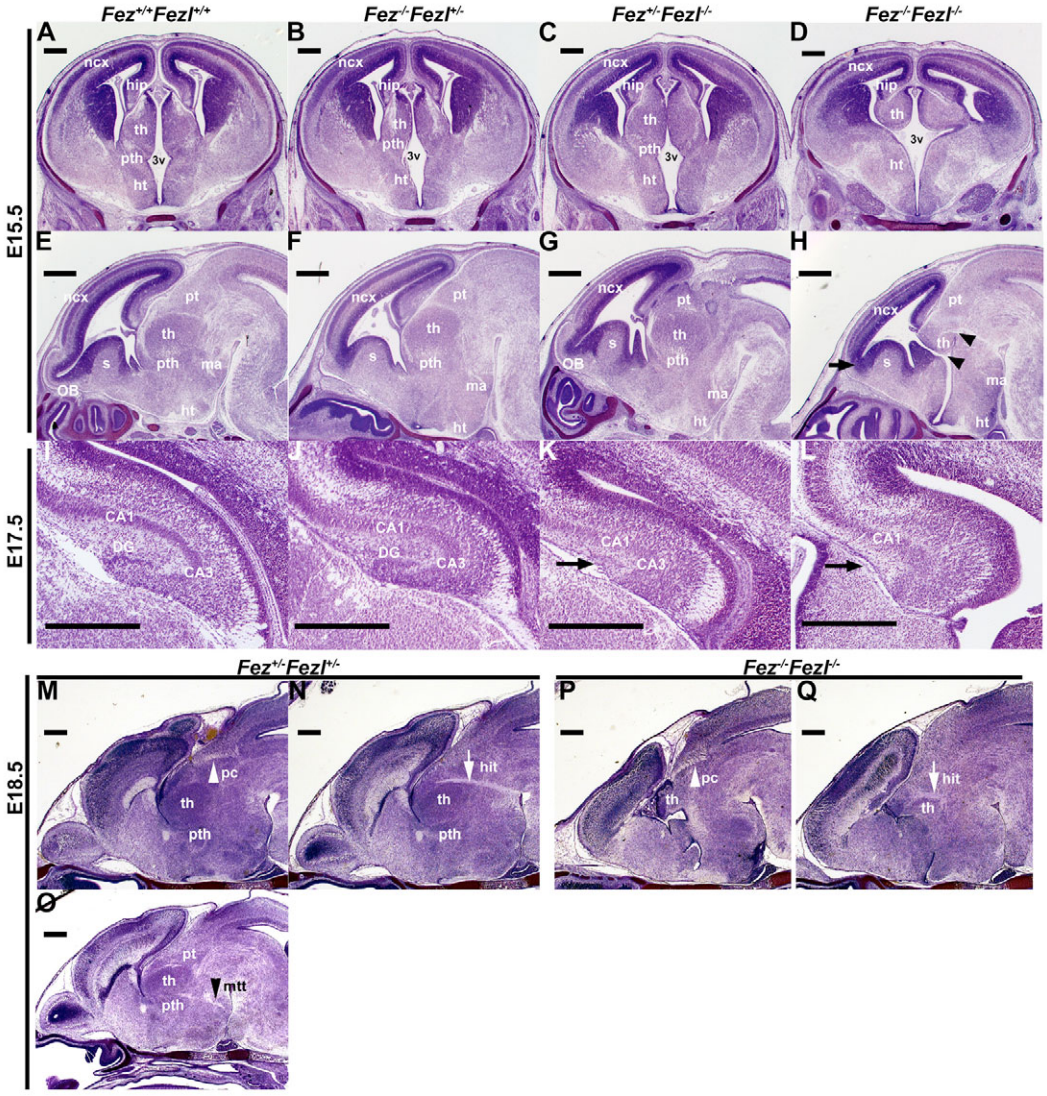

Fig. 1. Morphology of the forebrain of wild-type, $Fez^{-/-}Fezl^{+/-}$, $Fez^{+/-}Fezl^{-/-}$, $Fez^{-/-}Fezl^{-/-}$ and $Fez^{+/-}Fezl^{+/-}$ mice. Coronal (**A-D**,**I-L**) and sagittal (**E-H**) sections of E15.5 (A-H), E17.5 (I-L) wild-type (A,E,I), $Fez^{-/-}Fezl^{+/-}$ (B,F,J), $Fez^{+/-}Fezl^{-/-}$ (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 mammillthalamic 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 mammilothalamic tracts. DG, dentate gyrus; hit, habenulo-interpeduncular tract; hip, hippocampus; ht, hypothalamus; ma, mamillary region; mtt, mammilothalamic trancts; ncx, 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 tracts 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 mammilothalamic 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)

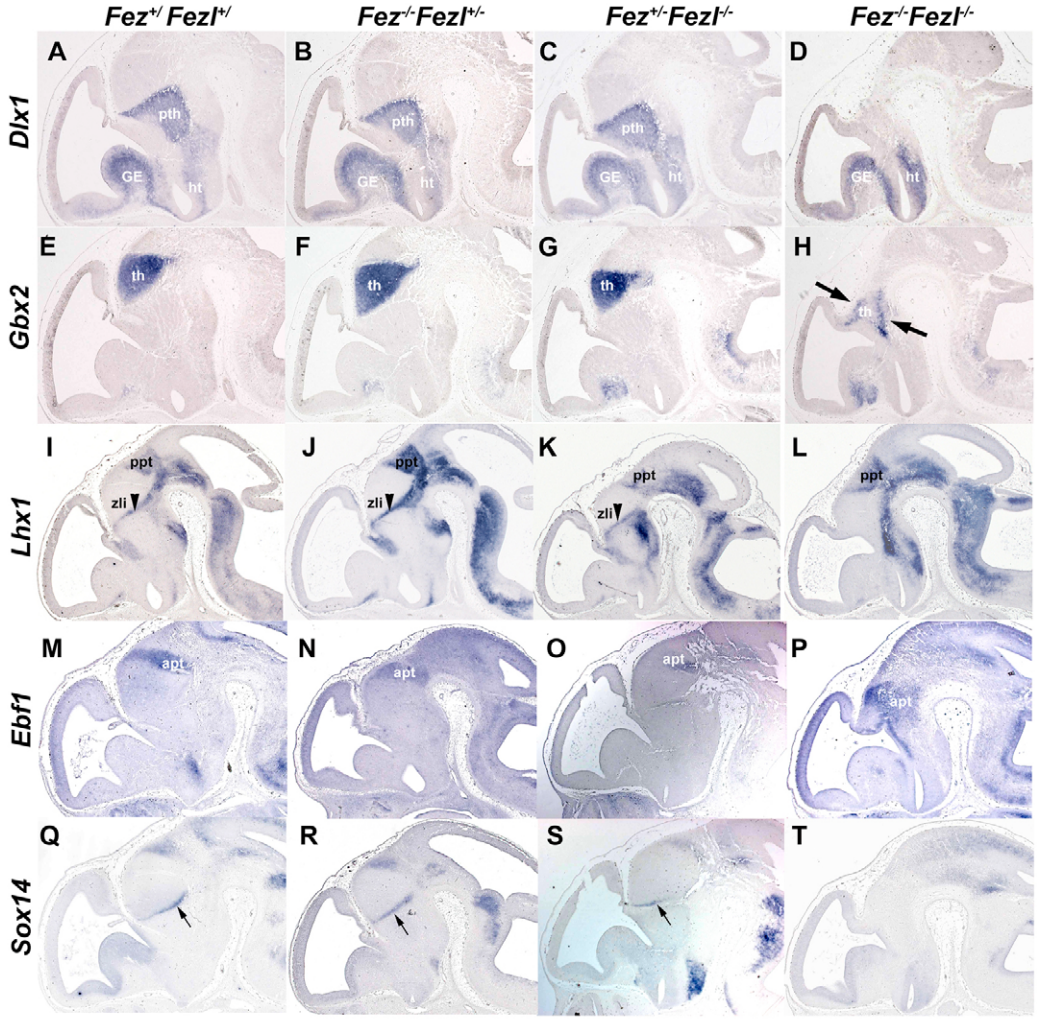

Fig. 2. Defects in prethalamus and thalamus development and rostral expansion of the pretectum in *Fez*$^{-/-}$*Fezl*$^{-/-}$ embryos at E12.5. (**A-T**) Expression of *Dlx1* (marker for prethalamus and ganglionic eminence, A-D), *Gbx2* (thalamus, E-H), *Lhx1* (posterior pretectum and ZLI, I-L), *Ebf1* (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.

(Bulfone et al., 1993; Stuhmer et al., 2002). 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-postive 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

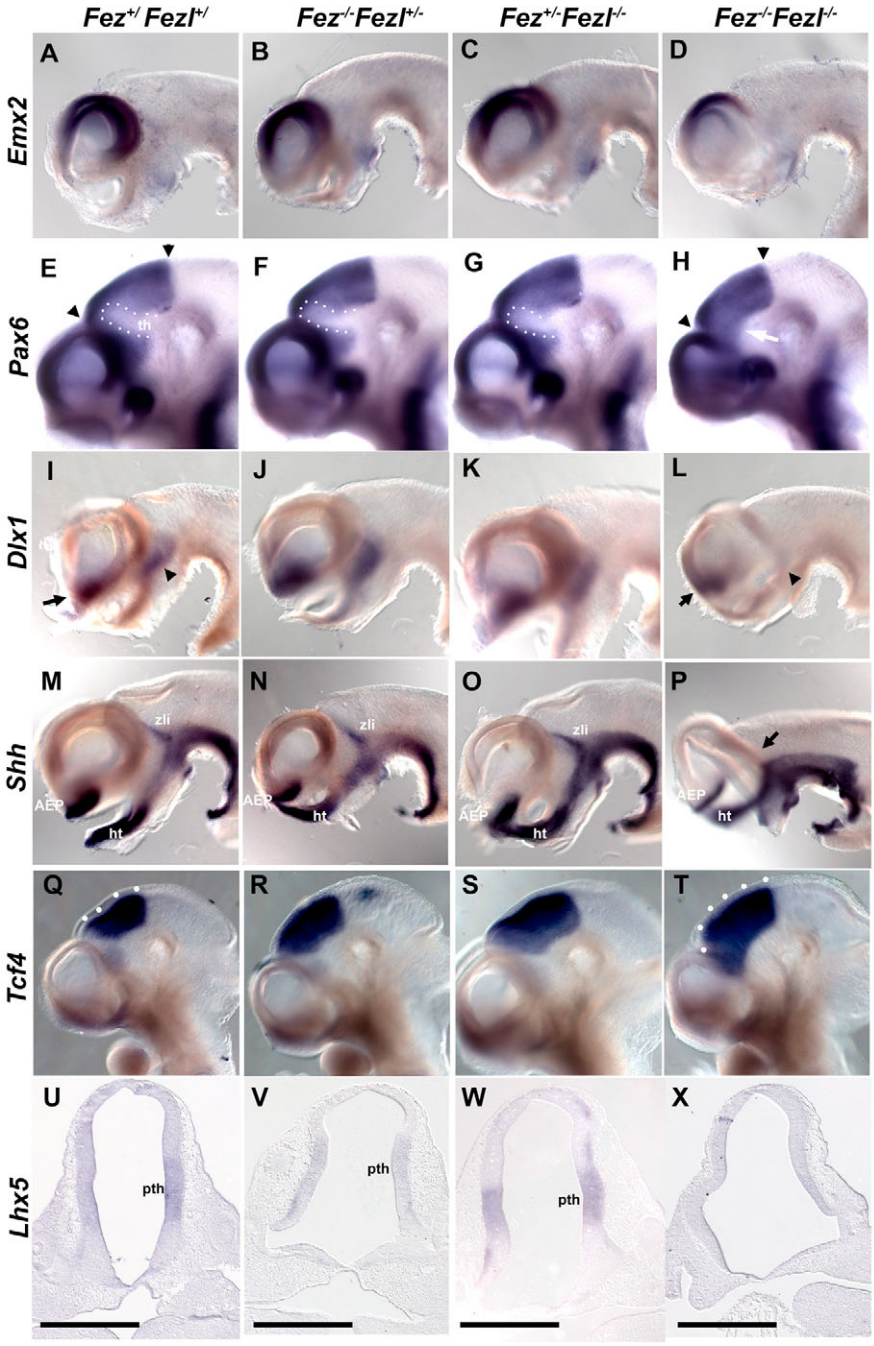

Fig. 3. Defects in telencephalon formation and regionalization of the diencephalon in *Fez*$^{-/-}$*Fezl*$^{-/-}$ embryos. Expression of *Emx2* (**A-D**), *Pax6* (**E-H**), *Dlx1* (**I-L**), *Shh* (**M-P**), *Tcf4* (**Q-T**) and *Lhx5* (**U-X**) in control (A,E,I,M,Q,U), *Fez*$^{-/-}$*Fezl*$^{+/-}$ (B,F,J,N,R,V), *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 et al., 1996; 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. *Shh* 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 pretectum (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 (eminentia 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,J) (Joyner and Martin, 1987). All these data indicate that the diencephalon patterning was already affected

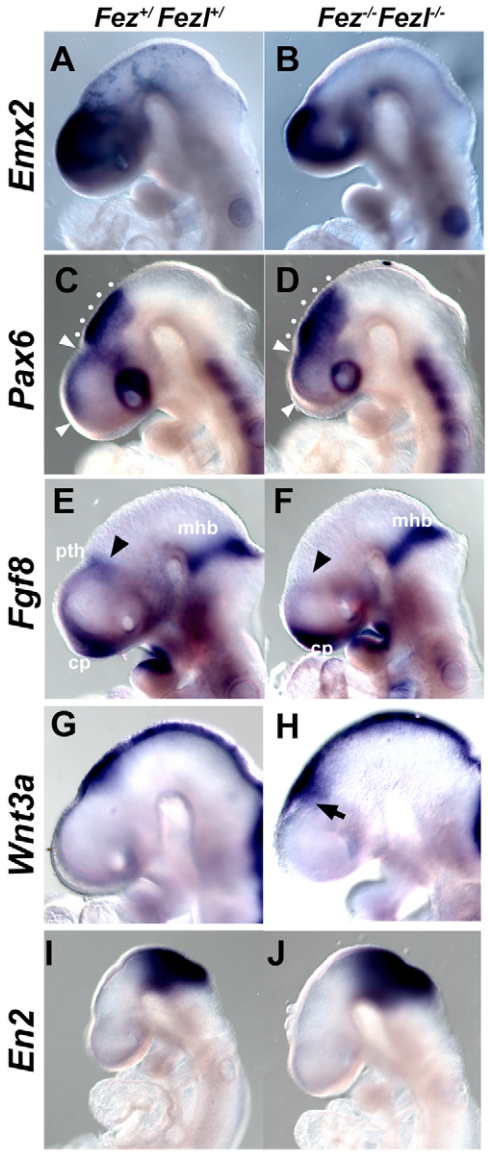

Fig. 4. Reduction of rostral diencephalon and expansion of caudal diencephalon in $Fez^{-/-}Fezl^{-/-}$ embryos at E9.5. Expression of *Emx2* (**A**,**B**), *Pax6* (**C**,**D**), *Fgf8* (**E**,**F**), *Wnt3a* (**G**,**H**) and *En2* (**I**,**J**) in control (A,C,E,G,I) and $Fez^{-/-}Fezl^{-/-}$ embryos (B,D,F,H,J) was analyzed by whole-mount in situ hybridization. Lateral views of anterior neuroectoderm, with anterior to the left. Expression of *Emx2* (A) and *Pax6* in the dorsal telencephalon (between arrowheads, C) was reduced, but the *Pax6* expression in the diencephalon (marked by dots) was maintained in $Fez^{-/-}Fezl^{-/-}$ embryos. *Fgf8* expression in the commissural plate and mid-hindbrain boundary was maintained, but that in the dorsal prethalamus was absent (arrowheads E,F) in $Fez^{-/-}Fezl^{-/-}$ embryos. The expression domain of *Wnt3a* was caudal to the ZLI in control embryos (G) and was expanded rostrally in $Fez^{-/-}Fezl^{-/-}$ embryos (arrow in H). Expression of *En2*, which marks the midbrain, was not affected in $Fez^{-/-}Fezl^{-/-}$ embryos (I,J). cp, commissural plate; mhb, mid-hindbrain boundary; pth, prethalamus.

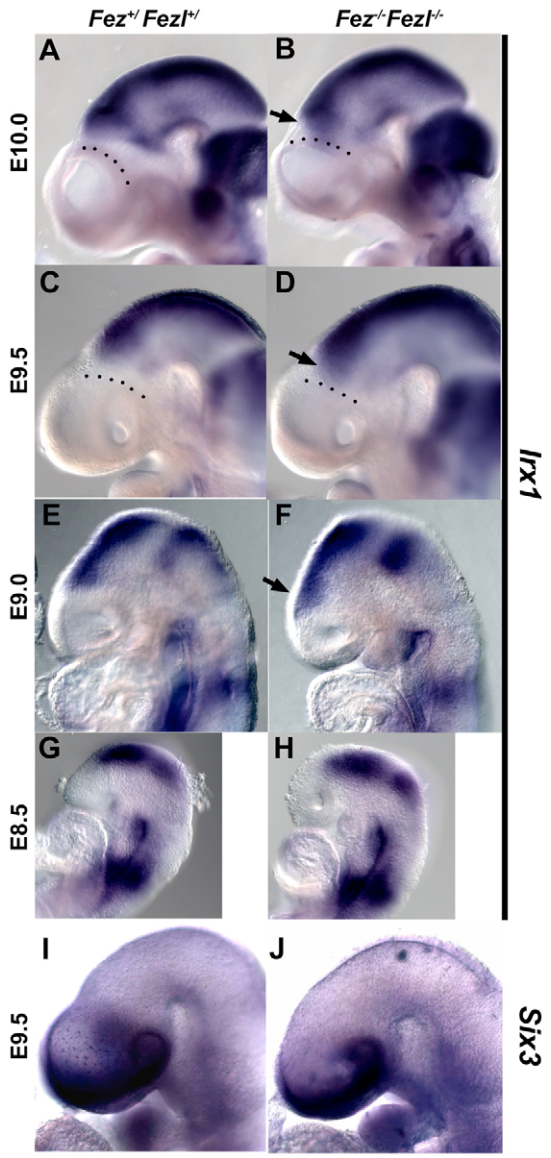

Fig. 5. Rostrally expanded expression of *Irx1* in *Fez*$^{-/-}$*Fezl*$^{-/-}$ embryos. *Irx1* and *Six3* expression in control (**A**,**C**,**E**,**G**,**I**) and *Fez*$^{-/-}$*Fezl*$^{-/-}$ (**B**,**D**,**F**,**H**,**J**) embryos at E10.0 (A,B), E9.5 (C,D,I,J), E9.0 (E,F) and E8.5 (G,H). Lateral views, with anterior to the right. *Irx1* expression was not significantly different between *Fez*$^{-/-}$*Fezl*$^{-/-}$ and control embryos at E8.5. A rostral expansion of *Irx1* expression was detected at E9.0 in *Fez*$^{-/-}$*Fezl*$^{-/-}$ embryos (arrow). At E9.5 and E10.5, the rostral limit of *Irx1* expression (arrows) reached the caudal edge of the telencephalon (marked by dots). Expression of *Six3* was not significantly affected in *Fez*$^{-/-}$*Fezl*$^{-/-}$ embryos at E8.5 (data not shown) and at E9.5 (I,J).

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 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. 5I,J, 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 *Fezl* 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 *Fezl* gene (Fig. 7A), and examined the *lacZ* expression in the resulting transgenics (Fig. 7B-D). The 8.2 kbp *Fezl* enhancer/promoter recapitulated the expression in the forebrain at E8.5 (Fig. 7B). By contrast, the 2.7 kbp *Fezl* enhancer/promoter (*Fezl2.7p*) 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* promoter-*lacZ*, *lacZ* activity was detected in a wider region than the endogenous *Fezl* expression (Fig. 7D), indicating that the enhancer/promoter could drive expression caudal to the ZLI. Using *Fezl2.7p*, we expressed *Fezl* or *Fez* cDNA ectopically in the caudal diencephalon. We monitored the exogenous *Fezl* or *Fez* expression with a green fluorescence protein variant, Venus (IRES-Venus) (Nagai et al., 2002). In embryos with the *Fezl2.7p-Fezl-IRES-Venus* or *Fezl2.7p-Fez-IRES-Venus* transgene, the expression of *Foxg1* in the telencephalon (Tao and Lai, 1992) was not affected, but the expression of *Irx1* was reduced and its rostral limit was shifted caudally at E9.5 (Fig. 8B,C,S). The rostral limit of *Irx1* corresponded to the caudal limit of the exogenous *Fezl-IRES-Venus* expression in these transgenic embryos (Fig. 8A,R). Similarly, in the *Fezl2.7p-Fezl-IRES-Venus* and *Fezl2.7p-Fez-IRES-Venus* transgenic embryos, the *Tcf4*-high domains, corresponding to the thalamus and pretectum, were strongly reduced at E10.5 (Fig. 8E,F,U). In transgenic embryos strongly expressing *Fezl*, *Dlx1* expression expanded caudally (*n*=1/2), with *Gbx2* expression being prominently reduced (*n*=2/3) at E12.5 (Fig. 8,M,N,P,Q). Furthermore, the misexpression of *Fezl* by *Fezl2.7p* abolished *Shh* expression in the ZLI and ventral diencephalon (*n*=2/4) or shifted the ZLI-specific *Shh* expression caudally (*n*=2/4) at E10.5 (Fig. 8H,I,K). The misexpression of *Fez* by *Fezl2.7p* abolished *Shh* expression in the ZLI (data not shown, *n*=1/1). We also used an *Otx2FM* enhancer, which can drive a transgene in the midbrain, diencephalon and archicortex (Kurokawa et al., 2004a), to misexpress *Fezl*. *Fezl* misexpression under this enhancer also

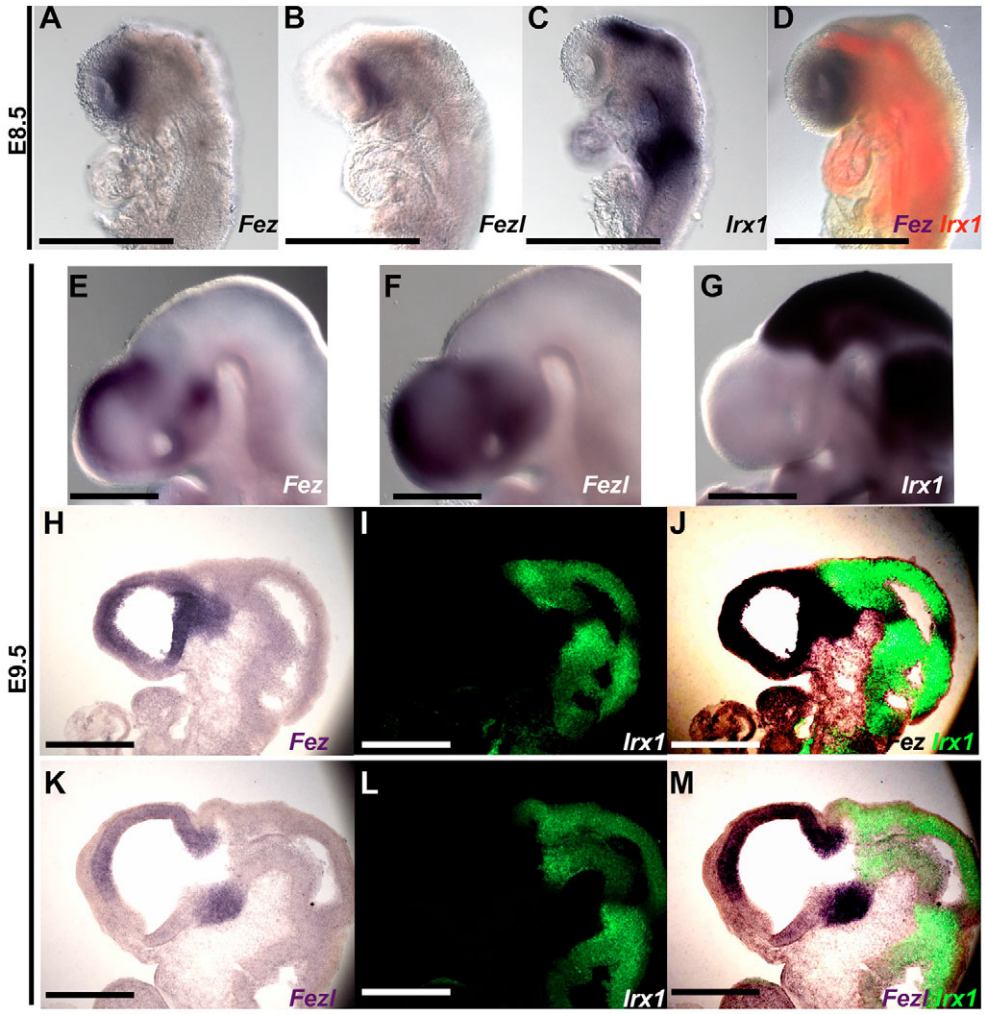

Fig. 6. Complementary expression of *Fez/Fezl* and *Irx1*. (**A-D**) Expression of *Fez* (A,D), *Fezl* (B) and *Irx1* (C,D) at E8.5. (D) Two-color staining. *Fez* and *Irx1* transcripts were stained with BM Purple and Fast Red; the fluorescence image from the Fast Red was superimposed on the bright-field image. (**E-M**) Expression of *Fez* (E), *Fezl* (F) and *Irx1* (G) at E9.5. (H-M) Sagittal sections of E9.5 embryos were hybridized with *Fez* and *Irx1* (H-J), or *Fezl* and *Irx1* probes (K-M). The hybridized signals were stained with BM Purple (*Fez* and *Fezl*) and Fluorescein (*Irx1*). Bright-field images (H,K), fluorescence images (I,L) and the bright-field and fluorescence superimposed images (J,M).

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 (Fig. 6) (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 co-repressors (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 Fezl function in this animal (Levkowitz et al., 2003), suggesting that FEZ and FEZL function as transcriptional repressors to regulate patterning of the diencephalon.

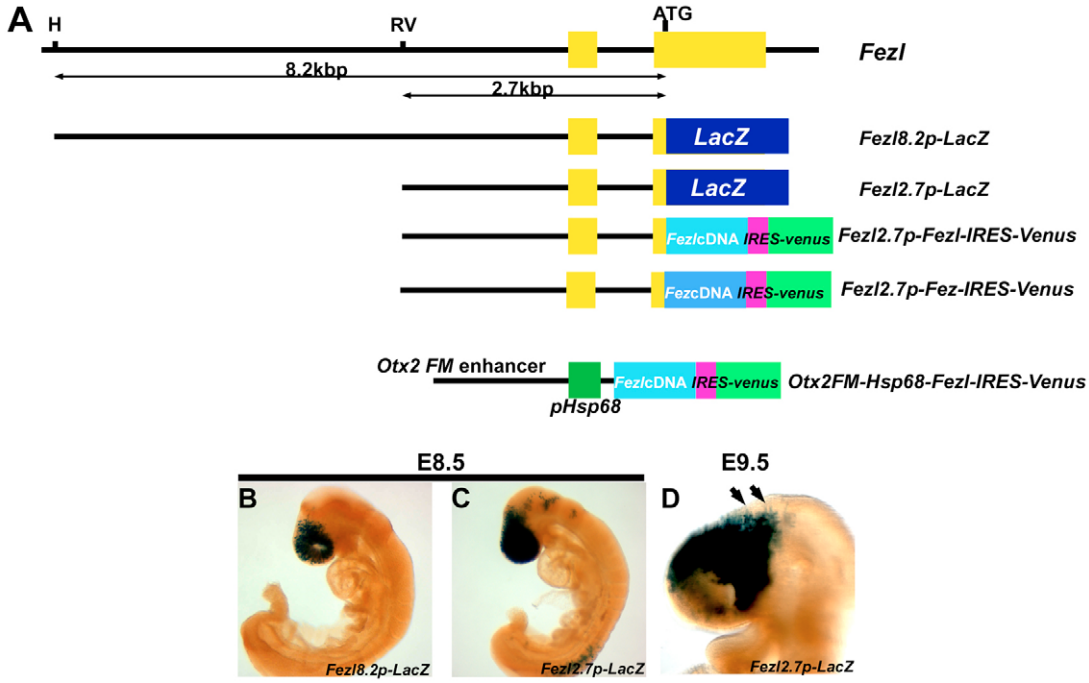

Fig. 7. Enhancer and promoter region of *Fezl*. (**A**) Schematic diagram of the *Fezl* enhancer/promoter and the constructs used for transgenesis. (**B-D**) Detection of *lacZ* expression by X-gal staining in *Fezl8.2p-lacZ* (B) and *Fezl2.7p-lacZ* (C,D) transgenic mouse embryos, in which *lacZ* expression was driven by the 8.2 kbp and 2.7 kbp *Fezl* enhancer/promoter, respectively. (B,C) E8.5 embryos, lateral views with anterior to the top. (D) E9.5 embryo, lateral views of the anterior neuroectoderm. The 8.2 kbp *Fezl* enhancer/promoter recapitulated the endogenous *Fezl* expression (rostral to the ZLI), whereas the 2.7 kbp *Fezl* enhancer/promoter showed a caudally expanded expression of *lacZ* (arrows, D).

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 combinatory gene disruption of *Six3* and *Fez* and/or *Fezl* will clarify this issue.

How *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, Emx1/2, 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

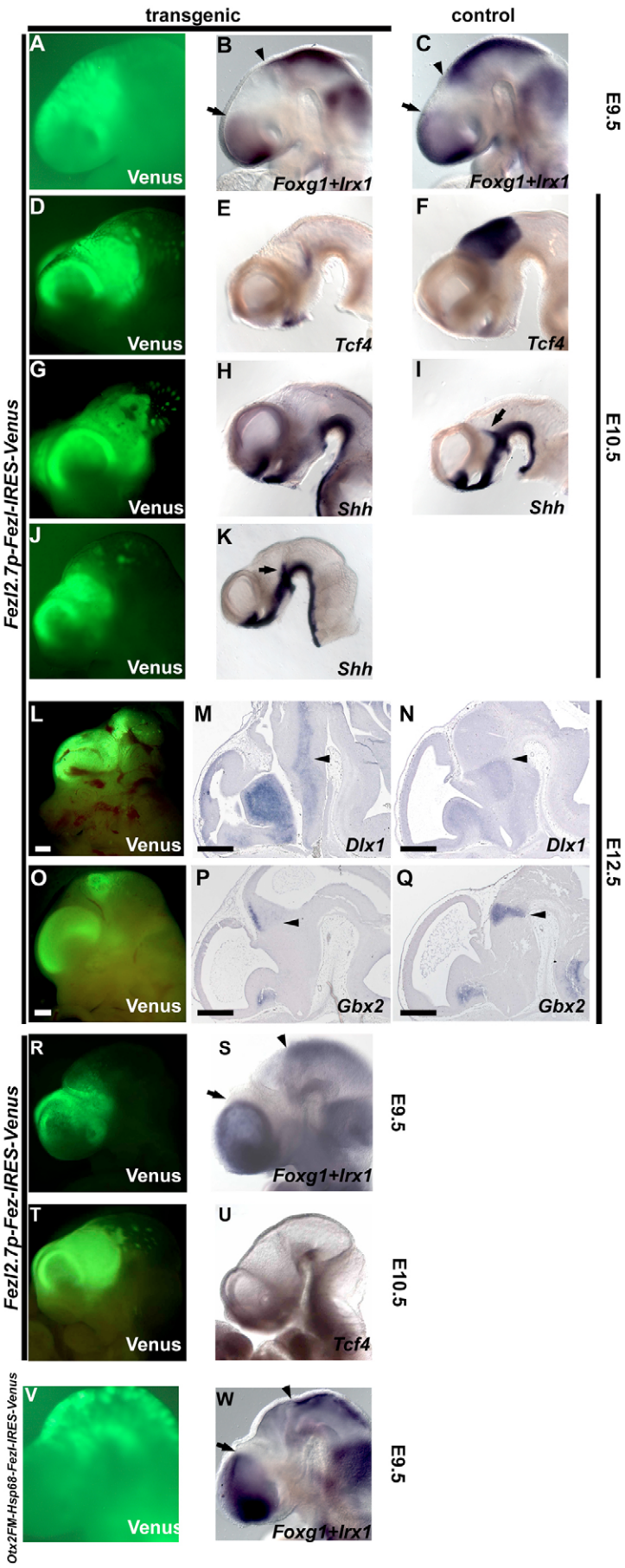

Fig. 8. Misexpression of *Fez* or *Fezl* affects rostro-caudal polarity in the diencephalon. Misexpression of *Fezl* (**A-Q**) or *Fez* (**R-U**) by the 2.7 kbp *Fezl* enhancer/promoter (*Fezl2.7p-Fezl-IRES-Venus, Fezl2.7p-Fez-IRES-Venus*), or *Fezl* by the FM enhancer of the *Otx2* gene and mouse *Hsp68* promoter (*Otx2FM-Hsp68-Fezl-IRES-Venus*, **V**,**W**) affected the diencephalon subdivisions. Exogenous *Fezl* 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,I,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-Fezl-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 *Fezl-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).

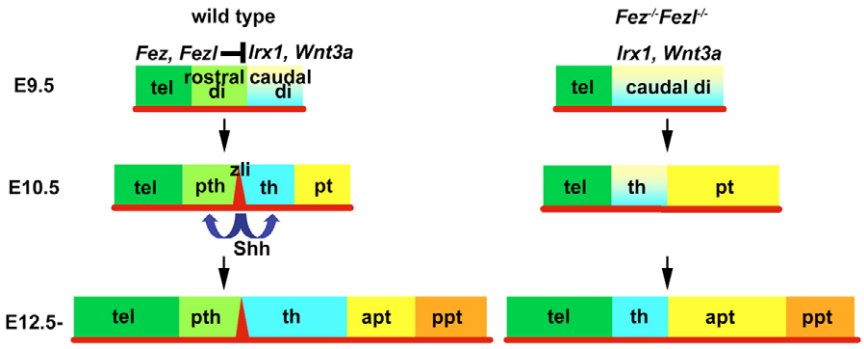

Fig. 9. Schematic presentation of a role of *Fez* and *Fezl* in diencephalon patterning. *Fez* and *Fezl* are expressed in the telencephalon and rostral diencephalon (prospective prethalamus) and function to suppress the formation of the caudal diencephalon, which expresses *Irx1* and *Wnt3a*. In the absence of *Fez* and *Fezl*, the rostral diencephalon does not form and instead caudal diencephalon expands rostrally at E9.5. Subsequently at E10.5, the prethalmaus and the ZLI, which is normally located in the interface between the prethalamus and thalamus, do not form in $Fez^{-/-}Fezl^{-/-}$ embryos. The caudal diencephalon, including the thalamus and pretectum, is expanded in $Fez^{-/-}Fezl^{-/-}$ embryos. The formation of thalamus, however, is dependent on inductive signals (e.g. Shh) from the ZLI. In $Fez^{-/-}Fezl^{-/-}$ embryos, the thalamus does not grow properly, but the anterior pretectum remains expanded at E12.5. apt, anterior pretectum; di, diencephalon; pth, prethalamus, ppt, posterior pretectum; tel, telencephalon; th, thalamus.

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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