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Patterning the zebrafish diencephalon by the conserved zinc-finger protein Fezl

Jae-Yeon Jeong[1], Zev Einhorn[1], Priya Mathur[1], Lishan Chen[1], Susie Lee[1], Koichi Kawakami[2] and Su Guo[1,*]

The forebrain constitutes the most anterior part of the central nervous system, and is functionally crucial and structurally conserved in all vertebrates. It includes the dorsally positioned telencephalon and eyes, the ventrally positioned hypothalamus, and the more caudally located diencephalon [from rostral to caudal: the prethalamus, the zona limitans intrathalamica (ZLI), the thalamus and the pretectum]. Although antagonizing Wnt proteins are known to establish the identity of the telencephalon and eyes, it is unclear how various subdivisions are established within the diencephalon – a complex integration center and relay station of the vertebrate brain. The conserved forebrain-specific zinc-finger-containing protein Fezl plays a crucial role in regulating neuronal differentiation in the vertebrate forebrain. Here, we report a new and essential role of zebrafish Fezl in establishing regional subdivisions within the diencephalon. First, reduced activity of *fezl* results in a deficit of the prethalamus and a corresponding expansion of the ZLI. Second, Gal4-UAS-mediated *fezl* overexpression in late gastrula is capable of expanding the prethalamus telencephalon and hypothalamus at the expense of the ZLI and other fore- and/or mid-brain regions. Such altered brain regionalization is preceded by the early downregulation of *wnt* expression in the prospective diencephalon. Finally, *fezl* overexpression is able to restore the anterior forebrain and downregulate *wnt* expression in Headless- and/or Tcf3 (also known as Tcf7l1a)-deficient embryos. Our findings reveal that Fezl is crucial for establishing regional subdivisions within the diencephalon and may also play a role in the development of the telencephalon and hypothalamus.

KEY WORDS: *fezl*, *too few*, Zebrafish, Brain patterning, Progenitor cells, Forebrain, Diencephalon, ZLI, Prethalamus, Thalamus, Pretectum, Zinc finger

INTRODUCTION

The vertebrate forebrain is a tremendously complex structure and carries out essential functions such as regulating emotions, learning and memory, and hormonal homeostasis. It arises from anterior neuroectoderm during gastrulation. By the end of somitogenesis, distinct subdivisions are morphologically visible. At least two sequential events are thought to occur that lead to the establishment of these distinct forebrain subdivisions (Kiecker and Lumsden, 2005; Rubenstein et al., 1998; Stern, 2001; Wilson and Houart, 2004): (1) the anterior neural tissue acquires a crude initial regional identity by avoiding exposure to caudalizing factors, such as Wnt and FGF proteins. This initial regionalization also establishes local organizing centers in the neural plate, such as the anterior neural border [ANB, also know as the anterior neural ridge (ANR)] and the midhindbrain boundary (MHB). (2) These local organizing centers further refine initial regional patterning and lead to the establishment of subdivisions that later give rise to various structures in the mature CNS.

Forebrain regionalization is best understood in the context of telencephalic development (Rallu et al., 2002; Wilson and Houart, 2004; Wilson and Rubenstein, 2000). It has been shown previously that the establishment of telencephalic identity requires local suppression of Wnt signaling – an evolutionarily conserved pathway that regulates diverse processes, including embryonic patterning, cell fate determination, cancer and synaptogenesis (Moon et al., 2002; Patapoutian and Reichardt, 2000). In zebrafish, the proper development of the telencephalon requires the secreted Wnt antagonist Tlc (Houart et al., 2002), the transcriptional repressor Headless and/or Tcf3 (also known as Tcf7l1a – Zebrafish Information Network) (Kim et al., 2000) and the Wnt-pathway scaffolding-protein Masterblind and/or Axin1 (Heisenberg et al., 2001).

Compared with the telencephalon, much less is known as to how various subdivisions within the diencephalon are established. Although the embryonic diencephalon has been proposed to have a segmental organization (Figdor and Stern, 1993; Puelles and Rubenstein, 2003), cell-lineage restriction boundaries are not apparent between some of the segments (Larsen et al., 2001). During embryogenesis, a transient boundary-like structure called the zona limitans intrathalamica (ZLI) is present between the prethalamus and the thalamus. This region divides the diencephalon into the anterior (the prethalamus) versus the posterior, or caudal (the thalamus and pretectum) territories (Kiecker and Lumsden, 2005). In chick, the ZLI is predicted by the absence of lunatic fringe and the presence of *Wnt8b* expression, and, starting from the mid-somitogenesis stage, is demarcated by the expression of *Shh*, which has been shown to play a role in the proper maturation of the vertebrate diencephalon (Kiecker and Lumsden, 2004; Scholpp et al., 2006). High levels of Wnt signaling have also been suggested to promote posterior diencephalic fates (Braun et al., 2003; Kiecker and Niehrs, 2001; Masai et al., 1997; Nordstrom et al., 2002). However, the genes and pathways that establish the ZLI and other diencephalic subdivisions are elusive. Based on mis-expression studies (Kobayashi et al., 2002), it has been proposed that cross-repression between the Irx family of homeodomain proteins – which are expressed in the prospective caudal diencephalon (including the thalamus and pretectum) and midbrain (Lecaudey et al., 2005) – and Six3 homeodomain transcription factors – which are detected early

[1]Programs in Developmental Biology, Neuroscience and Human Genetics, Department of Biopharmaceutical Sciences, University of California San Francisco, CA 94143-2811, USA. [2]Division of Molecular and Developmental Biology, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan.

*Author for correspondence (e-mail: su.guo@ucsf.edu)

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in the entire anterior forebrain anlage and later mainly in the optic stalk and eye regions (Seo et al., 1998) – may contribute to the establishment of the ZLI and other diencephalic subdivisions. However, *six3* (also known as six3a – Zebrafish Information Network) expression is dynamic and regresses rostrally as development progresses (Kobayashi et al., 2002; Seo et al., 1998), leaving the anterior diencephalic domain free of both irx gene family and *six3* expression.

The zebrafish *fezl* gene was identified as a zinc-finger-containing gene induced by Dkk1 (Hashimoto et al., 2000), a secreted antagonist of Wnt signaling (Glinka et al., 1998). *fezl* has a paralog named *fez*, and both genes exhibit remarkable evolutionary conservation from flies to men (Matsuo-Takasaki et al., 2000). Little is known about the role of the *Drosophila* Fezl protein. In mammals, *Fezl* and *Fez* have been shown to regulate cortical neuronal differentiation (Chen et al., 2005a; Chen et al., 2005b; Hirata et al., 2004; Molyneaux et al., 2005) and olfactory-bulb development (Hirata et al., 2006b), respectively, but their early roles in brain regionalization are unclear, probably as a result of their extensively overlapping expression patterns. In zebrafish, a hypomorphic allele of *fezl*, *too few* (*tof*), reveals its role in monoaminergic neuron development (Guo et al., 1999; Jeong et al., 2006; Levkowitz et al., 2003). Moreover, the expression of zebrafish *fezl* (but not *fez*) is detected in distinct domains of forebrain primordia in early zebrafish embryos (Hashimoto et al., 2000) (Fig. 1, and data not shown). These observations intrigued us to investigate whether *fezl* has a role in early forebrain regionalization.

Here, we report that *fezl* is expressed exclusively in the presumptive telencephalon, diencephalon and hypothalamus shortly after gastrulation. Reduced activity of *fezl* results in a deficit of the prethalamus and a corresponding anterior expansion of the ZLI. Gal4-UAS-mediated *fezl* overexpression in late gastrula is capable of expanding the prethalamus, optic stalk, telencephalon and hypothalamus at the expense of the eyes, ZLI and posterior fore- and/or mid-brain regions. The enlargement of these forebrain subdivisions is preceded by an early downregulation of *wnt* expression in the prospective diencephalon. Finally, *fezl* overexpression is able to restore anterior forebrain and downregulate *wnt1* expression in Headless- and/or Tcf3-deficient embryos. Our findings reveal a crucial role of Fezl in establishing regional subdivisions within the diencephalon, and also uncover the capability of Fezl in repressing Wnt proteins and in promoting the development of the telencephalon and hypothalamus.

MATERIALS AND METHODS

Establishment and analyses of transgenic zebrafish

The UAS-*fezl* transgene was cloned into the transposon vector pT2KXIG (Kawakami et al., 2004). The resulting plasmid DNA was injected, together with transposase RNA, into one-cell-stage embryos. Zebrafish embryos were raised at 28.5°C and staged according to Kimmel et al. (Kimmel et al., 1995). UAS-*fezl*-carrying transgenic founders were identified by PCR on pooled progeny and propagated by crossing with wild-type fish. UAS-*fezl* transgenic lines were subsequently crossed with *hsp-gal4* containing transgenic fish to yield double-transgenic embryos.

Morpholino designs and analyses of morphants

The sequences for *fezl* splicing-blocking morpholinos (MOs) and the effectiveness of MOs to block *fezl*-RNA splicing was as previously described (Jeong et al., 2006). Embryos were injected with 3-4 nl of 0.1 mM *fezl* sMO at the one-to four-cell stages. The *hdl/tcf3* MO was synthesized according to published information (Dorsky et al., 2003), and 3-4 nl of 0.3 mM was used per embryo.

RESULTS

fezl expression in the developing forebrain demarcates the prospective telencephalon, hypothalamus and prethalamus

fezl expression was first detected at ~75% epiboly exclusively in anterior neuroectoderm (Fig. 1A); by the tailbud stage, it was confined to the prospective telencephalon, hypothalamus and diencephalon (Fig. 1B). Its expression in these regions was maintained throughout the segmentation stages (Fig. 1C,D). The

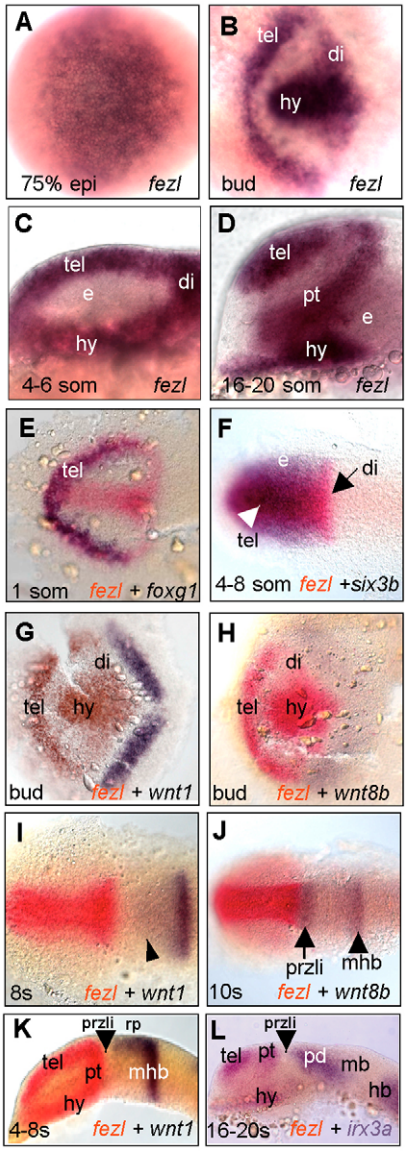

Fig. 1. *fezl* expression in the developing forebrain demarcates the prospective telencephalon, hypothalamus and prethalamus. (**A-L**) Analysis of gene expression. Arrow in F indicates the prospective diencephalon; arrowhead in F indicates the telencephalon; arrowhead in I indicates *wnt1* expression; arrow in J and arrowhead in K,L indicate the presumptive ZLI; arrowhead in J indicates the MHB. All are dorsal views except C,D,K,L, which are lateral views. Stages and marker identities are indicated in each panel. di, prospective diencephalon; e, eye region; hy, hypothalamus; hb, hindbrain; mhb, midhindbrain boundary; pd, posterior diencephalon; przli, presumptive ZLI; pt, prethalamus; tel, telencephalon; epi, epiboly; som, somite.

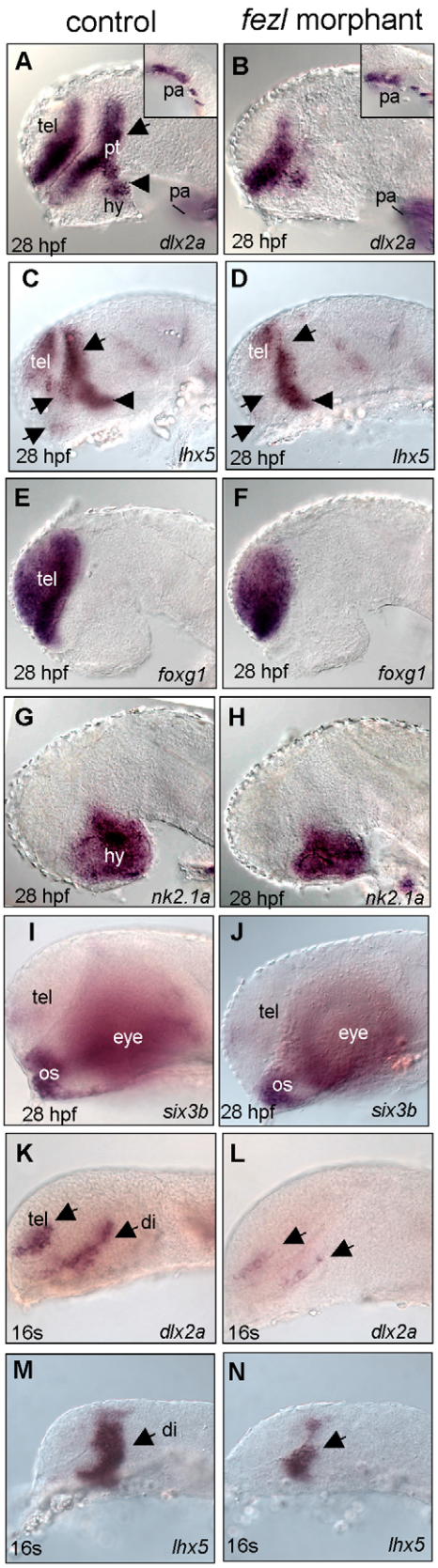

Fig. 2. Reduced *fezl* activity results in a deficit of the prethalamus. (**A**,**B**) Severe deficit of *dlx2a* in the prethalamus of a 28-hpf *fezl* morphant. Insets in A,B show unaffected *dlx2a* expression in the pharyngeal arch (pa) area. Arrow in A indicates *dlx2a*-expressing prethalamic cells; arrowhead in A indicates *dlx2a*-expressing hypothalamic cells. (**C**,**D**) Reduction of *lhx5* in the prethalamic region of the *fezl* morphant. Arrows in C,D indicate *lhx5*-expressing prethalamic cells; arrowhead in C,D indicates *lhx5*-expressing posterior tubercular cells. (**E-J**) Expression of *foxg1* in the telencephalon (E-F), *nk2.1a* in the hypothalamus (G-H) and *six3b* in the eye and optic stalk (I-J) are largely normal in *fezl* morphants. (**K-N**) Reduction of *dlx2a* and *lhx5* expressions in 16-somite (16s) *fezl* morphants. Arrows in K,L indicate the telencephalon and prospective diencephalon; arrow in M,N indicates the prospective diencephalon. All are lateral views with specific stages and marker identities indicted in each panel. di, prospective diencephalon; hy, hypothalamus; pt, prethalamus; tel, telencephalon; os, optic stalk.

expression of *fezl* overlapped with the telencephalic marker *foxg1* (Fig. 1E and see Fig. S1A in the supplementary material) and anterior forebrain marker *six3* (Fig. 1F and data not shown, and see Fig. S1B in the supplementary material) but not with the eye field marker *rx3* (see Fig. S1C,D in the supplementary material). During early somitogenesis, whereas *six3* and *fezl* expression overlapped in the prospective telencephalon, only *fezl* expression was detected in the prospective diencephalon (Fig. 1F and see Fig. S1E,F in the supplementary material). By late somitogenesis stages, *fezl* was strongly expressed in the dorsal telencephalon, prethalamus and hypothalamus (Fig. 1D), whereas *six3* expression was largely confined to the optic stalk and eye region (see Fig. 1G,H in the supplementary material). Compared with posteriorly expressed genes, the *fezl* domain at the tailbud stage was separated from that of *wnt1* and *wnt8b* by an approximately two- to three-cell diameter space; at least part of this area may represent the presumptive ZLI (Fig. 1G,H). By early somitogenesis, the *fezl*-expressing domain was separated by a gap (the presumptive ZLI) from *wnt1*-expressing cells in the roof plate and MHB (Fig. 1I,K), and from *irx3a*-expressing cells in the posterior diencephalon (Fig. 1L); moreover, *fez1* expression abutted *wnt8b* expression in the prospective ZLI (Fig. 1J). These analyses indicate that *fezl* expression is initiated early and exclusively in the developing forebrain, and is subsequently maintained in discrete forebrain subdivisions throughout the somitogenesis stages. These observations led us to hypothesize that *fezl* might have a role in forebrain regionalization.

Reduced *fezl* activity results in a deficit of the prethalamus

To determine the role of Fezl in forebrain development, we knocked-down *fezl* activity with two specific splicing morpholinos (MOs) (Jeong et al., 2006): both MOs gave similar results, whereas the control MOs had no effect. *fezl* MO-injected embryos (hereafter referred to as morphants) were examined at ~28 hours post fertilization (hpf), when brain subdivisions are distinct by morphology and gene expression; they were also examined at earlier stages (neural-plate and mid-somitogenesis) in order to better define the timing of *fezl* action.

In ~28 hpf *fezl* morphants, we found that *dlx2a* (Akimenko et al., 1994) expression in the prethalamus was significantly reduced in a dose-dependent fashion (Fig. 2B, 97%, n=65; and data not shown), while remaining unaffected in the pharyngeal arch progenitors (insets of Fig. 2B). In addition to *dlx2a*, the expression of *lhx5* (Peng and Westerfield, 2006; Scholpp et al., 2006; Toyama et al., 1995) in the prethalamus region was significantly reduced, but its expression in the posterior tubercular area remained largely normal (Fig. 2D, 90%,

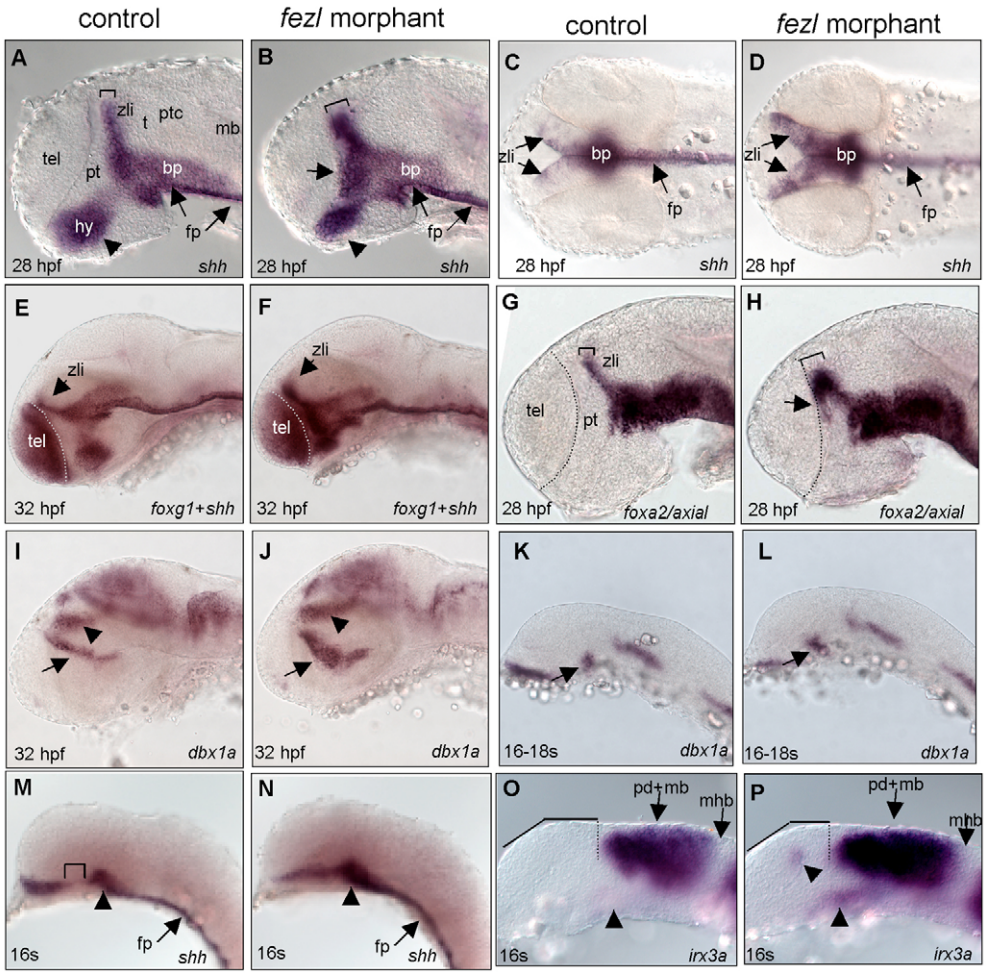

Fig. 3. The *fezl* morphants exhibit anterior expansion of the ZLI. (**A-D**) Marked anterior expansion of *shh*-expressing ZLI (B,D) was observed in *fezl* morphants. Arrows in A,B indicate the floor plate; arrowhead in A,B indicates hypothalamus; square brackets indicate the ZLI. (**E**,**F**) Double labeling of *foxg1* (a telencephalic marker) and *shh*, showing the expression of *shh* in the prethalamic area in the *fezl* morphant. (**G**,**H**) Anterior expansion of *foxa2* expression in the *fezl* morphant. Arrow in H indicates the expansion of *foxa2*-expressing cells; square brackets indicate the ZLI. (**I**,**J**) Expanded expression of *dbx1a* near the ZLI (arrow) and in the thalamus (arrowhead) in the *fezl* morphant. (**K-P**) *dbx1a, shh* and *irx3a* expression in the 16-somite-stage (16s) control and *fezl* morphants. Arrows in I-L indicate *lhx5*-expressing cells in the posterior tubercular region, and in M-N indicate the floor plate; arrowhead in I,J indicates *lhx5*-expressing cells in the thalamus, and in M-P indicates expanding *shh* expression in the presumptive ZLI; square bracket in M indicates the ZLI; dotted line in O,P indicates the anterior boundary of *irx3a* expression in the posterior diencephalon and midbrain region. All are lateral views of embryonic brains, except C,D, which are dorsal views. t, thalamus; hy, hypothalamus; mb, midbrain; ptc, pretectum; tel, telencephalon; fp, floor plate; bp, basal plate.

n=58). Despite the strong expression of *fezl* in the telencephalon and hypothalamus (Fig. 1B,D), expression of the telencephalic *foxg1* (Fig. 2F, 98%, *n*=60) and the hypothalamic *nk2.1a* (also known as *titf1a* – Zebrafish Information Network) (Fig. 2H, 98%, *n*=81) appeared largely normal (perhaps slightly reduced); the expression of *six3b* also appeared largely normal (Fig. 2J, 88%, *n*=33).

Analyses of early-stage *fezl* morphants revealed largely normal patterning at the neural-plate stage (data not shown, and see Fig. 2 in the supplementary material). However, by mid-somitogenesis, the expression of both *dlx2a* (Fig. 2L, 90%, *n*=119) and *lhx5* (Fig. 2N, 85%, *n*=44) in the prospective anterior diencephalon was significantly reduced, and *dlx2a* expression in the prospective telencephalon was also reduced at this early stage (Fig. 2L, 81%, *n*=119), suggesting a transient and later-recoverable telencephalic defect in the *fezl* morphants, possibly due to genetic compensation by other anterior forebrain factors (see Discussion). Together, these results indicate that *fezl* is essential for the development of anterior diencephalon (the prethalamus), starting at around mid-somitogenesis.

A corresponding anterior expansion of ZLI accompanies the loss of prethalamus in the *fezl* morphants

Since proliferation and cell death were not significantly affected in the *fezl* morphants (data not shown), we next asked whether the prethalamic defects might be a result of altered regionalization within the diencephalon. We examined the expression of *shha* (also known as *shh* – Zebrafish Information Network, and hereafter referred to as *shh*) and *foxa2*, two markers of the ZLI, which is located immediately posterior to the prethalamus. In control embryos, a gap occupied by the prethalamus existed between *shh*-expressing cells and the telencephalic boundary (Fig. 3A,C,E), but

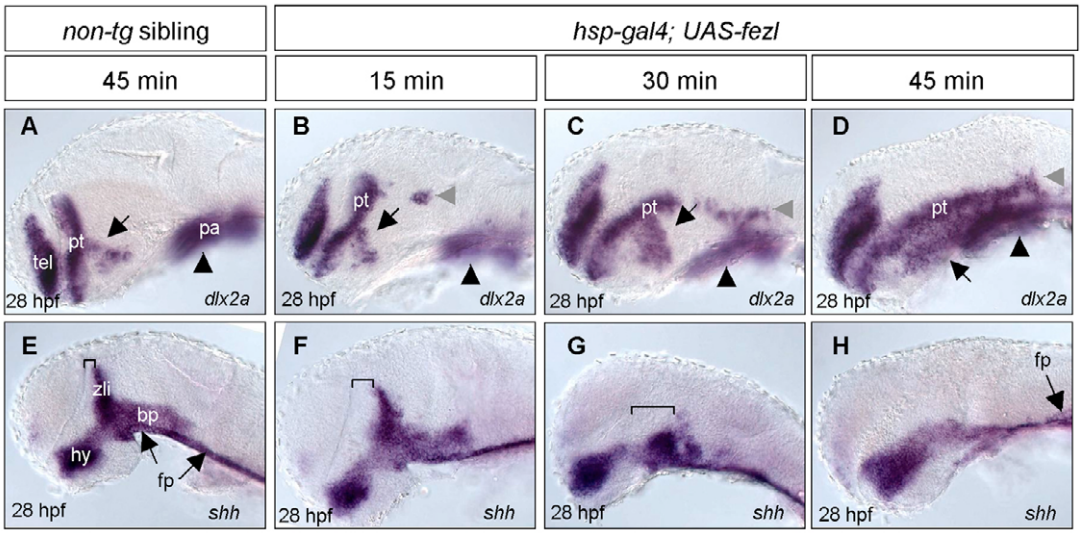

Fig. 4. Overexpression of *fezl* results in an expansion of the prethalamus and the elimination of the ZLI in a dose-dependent fashion. The *hsp-gal4* transgenic fish are crossed with *uas-fezl* transgenic fish and embryos are heat shocked at 37°C for 15-45 minutes at ~75% epiboly. Embryos are stained with indicated probes and analyzed at 28 hpf. Genotypes are identified by PCR with DNA from the tail. (**A-D**) The expression of *dlx2a* in the prethalamus is most-dramatically expanded. Its expression in the ventral telencephalon is mildly increased whereas its expression in the pharyngeal arches (pa) is not significantly altered. Arrow in A-D indicates *dlx2a*-expressing hypothalamic cells; black arrowheads in A-D indicate the pharyngeal arches; gray arrowhead in B-D indicates ectopic *dlx2a*-expressing cells in the midbrain. (**E-H**) The ZLI, as shown with *shh* expression, is moved caudally or completely eliminated depending on the duration of heat shock, but *shh* expression in the floor plate (fp) is unaffected. Square brackets indicate the ZLI. All are lateral views. hy, hypothalamus; pt, prethalamus; tel, telencephalon; fp, floor plate; bp, basal plate; pa, pharyngeal arches.

in ~28 hpf *fezl* morphants, this gap was filled in by expanded *shh* expression (Fig. 3B,D, 99%, *n*=83; Fig. 3F, 98%, *n*=46). The expression of *foxa2* was also expanded to abut the telencephalon (Fig. 3H, 95%, *n*=86), but *foxa2* expansion was noticeably most pronounced in the dorsal region, suggesting perhaps an easier transformation of the dorsal area as compared with the more ventral territory of the prethalamus. In addition to *shh* and *foxa2*, the expression of *dbx1a* – which is detected in the anterior diencephalon adjacent to or overlapping with the ZLI, and also in the posterior diencephalon (the thalamus) (Scholpp et al., 2006) – was significantly expanded in the anterior diencephalon (Fig. 3J, arrow, 90%, *n*=40), and moderately increased in the thalamic region (Fig. 3J, arrowhead, 90%, *n*=40). These analyses suggest that *fezl* activity is required to repress the fate of ZLI and possibly also the posterior diencephalon.

It has been previously reported that a slight dorsal extension of *shh* expression at early- to mid-somitogenesis stages probably demarcates the location of the future ZLI, and *dbx1a* expression is detected ventrally adjacent to the ZLI at this early stage (Scholpp et al., 2006). In ~16-somite stage *fezl* morphants, *dbx1a* expression adjacent to the ZLI appeared slightly increased (Fig. 3L, 85%, *n*=42); moreover, an increased and anteriorly expanded *shh* expression in the prospective ZLI was readily discernible, which was accompanied by the loss of the gap between the hypothalamic- and ZLI-expression, whereas its expression in the anterior hypothalamus was moderately decreased (Fig. 3N, 88%, *n*=50). *irx3a* expression, which is in the prospective caudal diencephalon and midbrain and in close proximity to *shh*-expressing prospective ZLI cells (Lecaudey et al., 2005), was moderately expanded anteriorly; enhanced *irx3a* expression was observed in a ventral location that might be overlapping with the *shh*-expressing prospective ZLI cells; in addition, a small cluster of *irx3a*$^{+}$ cells was detected in the anterior forebrain, possibly in the prospective telencephalon or in the dorsal prethalamus (Fig. 3P, 99%, *n*=40). This anterior forebrain expression appeared to be transient and might represent an incomplete fate switch because it was not observed in ~28 hpf *fezl* morphants (data not shown), and because other thalamic markers, such as *dbx1a*, were not detected in the corresponding location (data not shown). These results suggest that *fezl* plays a role in repressing the early ZLI and posterior diencephalic fate, beginning around the mid-somitogenesis stage.

Mis-expression of *fezl* results in expansion of the prethalamus and elimination of the ZLI

To further elucidate the role of *fezl* in forebrain regionalization, we investigated the consequence of overexpressing *fezl*. As the delivery of *fezl* mRNA into one- to four-cell-stage embryos led to severe embryonic deformity (data not shown) (Levkowitz et al., 2003; Yang et al., 2001), we employed the heat inducible Gal4-UAS system (Scheer and Camnos-Ortega, 1999) to achieve a temporal control of *fezl* overexpression. We established transgenic lines carrying the *UAS-fezl* transgene and crossed them with another line carrying the *hsp-gal4* transgene. The embryos derived from this cross were subjected to heat shock for 15, 30 or 45 minutes at ~75% epiboly, analyzed for gene expression at ~28 hpf, and subsequently genotyped to determine the presence or absence of the transgenes. Heat shock at later stages (e.g. 10-somite and 24 hpf) had little or no effect on brain regionalization (data not shown). Because the earliest time when the endogenous *fezl* expression was observed is at ~75% epiboly, our analyses were focused on the embryos that were heat shocked at this stage.

Ubiquitous *fezl* expression was detected ~2 hours post heat-shock through to 6 hours (Fig. S3 in the supplementary material). In the heat-shocked double-transgenic embryos, prethalamic and hypothalamic *dlx2a* expression was expanded in proportion to the duration of heat shock, whereas pharyngeal arch *dlx2a* expression was relatively normal (Fig. 4A-D, *n*=62). To determine whether the expansion of *dlx2a*-expressing domains might be at the expense of more posterior brain tissues, we examined the expression of *shh*. Remarkably, *shh*-expressing ZLI was shifted posteriorly, reduced or eliminated in the double-transgenic embryos in proportion to the duration of heat shock, whereas the floor plate expression of *shh* was unaffected (Fig. 4E-H, *n*=34). These analyses indicate that *fezl* overexpression is capable of expanding the prethalamus at the expense of the ZLI.

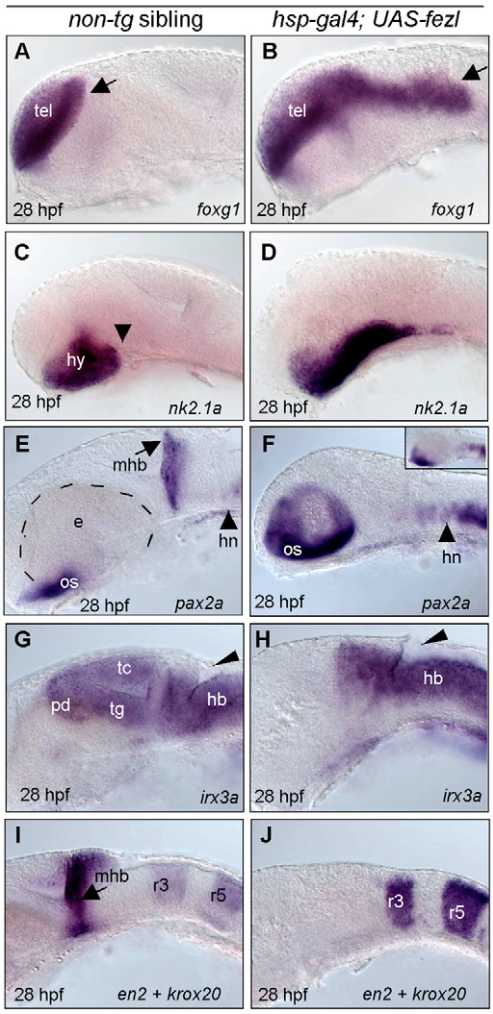

Fig. 5. Overexpression of *fezl* expands the telencephalon and hypothalamus at the expense of other fore- and mid-brain regions. (**A-D**) Expansion of telencephalic *foxg1* (B) and hypothalamic *nk2.1a* (D), as compared with wild type (A,C). Arrow in A,B indicates *foxg1*-expressing cells; arrowhead in C indicates posterior boundary of *nk2.1a*-expressing cells. (**E**,**F**) Expansion of the optic stalk (os), reduction of the eyes and loss of MHB in *fezl*-overexpressing embryos (F) compared with wild-type (E). Inset in F shows another embryo of the same genotype as that shown in F, with a less-dramatic expansion of optic stalk but missing *pax2a*-expressing midbrain cells. (**G-J**) Loss of caudal diencephalic and midbrain *irx3a* (H) and MHB *eng2* (J) expression in *fezl*-overexpressing embryos. Arrowhead in G,H indicates the mid- hind-brain boundary. All are lateral views of embryonic brain regions labeled with various region-specific markers. Stages and marker identities are specified in each panel. hy, hypothalamus; mhb, mid-hind-brain boundary; tel, telencephalon; os, optic stalk; e, eye region; tc, tectum; hb, hindbrain; pd, posterior diencephalon; tg, tegmentum.

Mis-expression of *fezl* expands the telencephalon and hypothalamus at the expense of other fore- and mid-brain regions

To further characterize the *fezl* gain-of-function (GOF) phenotype, we assessed the extent to which *fezl* overexpression could alter brain regionalization by examining additional region-specific genes: the expression of *foxg1* (Fig. 5B, *n*=24) in the telencephalon and *nk2.1a* in the hypothalamus (Fig. 5D, *n*=42), as well as *pax2a* in the optic stalk (Fig. 5F, *n*=10), were all expanded; by contrast, the expression of *pax2a* (Fig. 5F, *n*=10) and *engrailed 2* (also known as *engrailed 2a* – Zebrafish Information Network) (Fig. 5J, *n*=22) in the MHB, and *irx3a* in the caudal diencephalon and midbrain (Fig. 5H, *n*=10) were lost, and the eyes were significantly reduced (Fig. 5F, *n*=10). However, *krox-20* (also known as egr2a – Zebrafish Information Network) expression in the hindbrain rhombomeres r3 and r5 remained (Fig. 5J, *n*=22). Therefore, it appears that ectopic expression of *fezl* at late gastrulation can expand the telencephalon, hypothalamus and prethalamus at the expense of the eyes, ZLI, caudal diencephalon, midbrain and MHB.

fezl gain-of-function embryos exhibit a selective downregulation of *wnt1* and *pax2a* at the tailbud stage, and deficits of ZLI and MHB by mid-somitogenesis

To understand better the underlying cause of the *fezl* GOF phenotype, we examined *fezl* overexpressing embryos at early stages. At the tailbud stage, the brain subdivisions are not yet morphologically discernible, but the Wnt antagonist Tlc and transcription factors Emx1 and Foxg1 are expressed at the anterior margin of the neural plate, overlapping with the expression of Fezl (Fig. 1E and Fig. 6A,C; and data not shown). Both *wnt1* and *wnt8b* are expressed in the presumptive caudal diencephalon (Fig. 1G,H). In tailbud-stage *fezl*-overexpressing embryos, the expression of *emx1*, *tlc*, *foxg1*, *six3b*, *wnt8b*, *irx3a* and *fgf8* was unaffected (Fig. 6A-H,O-R; and data not shown). However, the expression of *wnt1* (Fig. 6J,N, *n*=34) and *pax2a* (Fig. 6L,N,P, *n*=38) was severely deficient. By the mid-somitogenesis stage, the expression of *wnt8b* in the prospective ZLI and MHB (Fig. 6V, *n*=19) and *fgf8* in the MHB (Fig. 6X, *n*=16) was found reduced, and the expression of telencephalic *fgf8*, *six3b*, *emx1* and *foxg1* was found extended caudally (Fig. 6T,X; and data not shown). These observations reveal that the earliest effect of *fezl* overexpression is an inhibition of *wnt1* and *pax2a* at the neural-plate stage, followed by deficits of two important organizers – the ZLI and MHB – by the mid-somitogenesis stage. These effects could underlie the GOF phenotypes observed at ~28 hpf.

fezl overexpression is sufficient to restore anterior forebrain gene expression in Headless- and/or Tcf3-deficient embryos

headless and/or *tcf3* (*hdl/tcf3*), which encode a transcriptional repressor of Wnt target genes (*headless* being the mutant form of *tcf3*), has been shown previously to be crucial for the development of the forebrain: its inactivation leads to a headless phenotype and a dramatic expansion of *wnt1* expression into anterior forebrain (Kim et al., 2000). Injection of *six3* mRNA, a known repressor of *wnt1* transcription, into Hdl/Tcf3-deficient embryos has been shown to rescue the eyes, as well as the head, based on gross morphology, but the extent of head rescue by *six3* has not been examined at the molecular level (Lagutin et al., 2003).

We reasoned that, if Fezl were a crucial player in establishing forebrain subdivisions, overexpression of *fezl* should restore these subdivisions to Hdl/Tcf3-deficient embryos. Because the maternal zygotic *hdl/tcf3* mutant had a rather variable severity of head loss (our unpublished observations), we delivered the *hdl/tcf3* MO, which was previously shown to be effective and specific in knocking down *hdl/tcf3* activity (Dorsky et al., 2003; Dorsky et al., 2002), to embryos derived from the *hsp-gal4/+*×*uas-fezl* cross. These *hdl/tcf3* morphants were subjected to heat shock at ~75% epiboly, analyzed

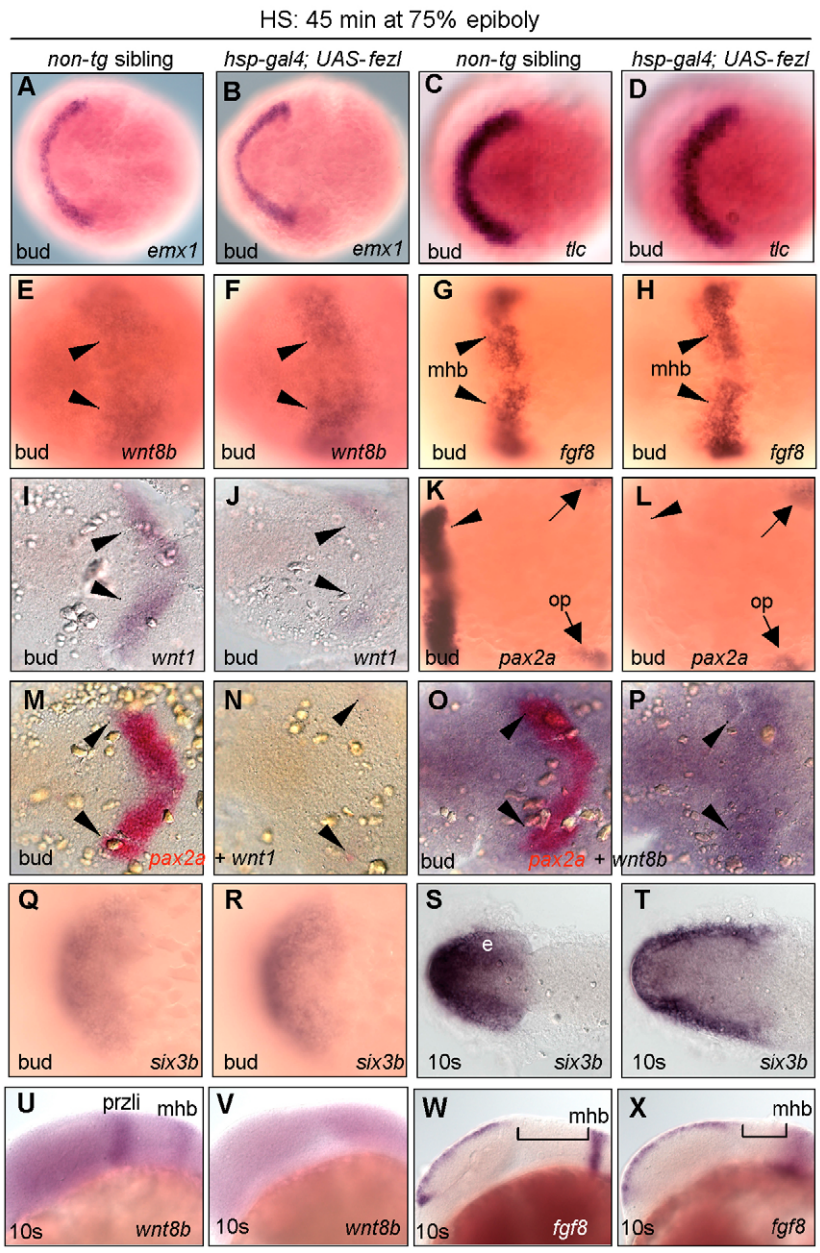

Fig. 6. Downregulation of *wnt* expression in early-stage *fezl*-overexpressing embryos. (**A-R**) Bud-stage embryos. The expressions of *emx1* (B), *tlc* (D), *wnt8b* (F,P), *fgf8* (H) and *six3b* (R) were unaffected in *fezl*-overexpressing embryos at the bud stage, whereas the expression of *wnt1* (J,N) and *pax2a* (L,N,P) was defective in bud-stage *fezl*-overexpressing embryos. Arrowheads in A-P indicate the MHB; arrows in K,L indicate the otic placode. (**S-X**) 10-somite-stage (10s) embryos showing altered expressions of *six3b* (T), *wnt8b* (V) and *fgf8* (X). All are dorsal views of the embryonic brain anlages. Stages and marker identities are specified in each panel. op, otic placode; e, eye region.

for forebrain gene expression at ~28 hpf, and subsequently genotyped for the presence or absence of the transgenes. At the 3-somite stage, *fezl* expression was dramatically reduced and *wnt1* expression was significantly increased in the *hdl/tcf3* morphants (Fig. 7A,B, *n*=43). *fezl* overexpression was sufficient to repress *wnt1* expression in the *hdl/tcf3* morphant (Fig. 7C, 81%, *n*=36). Similarly, whereas the lack of *hdl/tcf3* activity led to severe deficits in the expression of *foxg1* (Fig. 7D, 62%, *n*=52), *dlx2a* (Fig. 7G, 67%, *n*=42) and *nk2.1a* (Fig. 7J, 45%, *n*=29) in anterior brain regions, *fezl* overexpression was not only able to restore, but was also able to expand, the telencephalic *foxg1* expression (Fig. 7F, 80%, *n*=35), prethalamic *dlx2a* expression (Fig. 7I, 91%, *n*=34) and hypothalamic *nk2.1a* expression (Fig. 7L, 84%, *n*=13) in the *hdl/tcf3* morphants. These analyses demonstrate that Fezl overexpression is sufficient to repress *wnt1* and to promote telencephalic hypothalamic and prethalamic identity even in the absence of the *wnt* signaling repressor Hdl/Tcf3.

DISCUSSION

The establishment of subdivisions is a crucial early step towards the formation of stereotyped neuronal patterns, intricate neural connectivity and subsequent functioning of the vertebrate forebrain. Through loss-of-function (LOF) studies, we have revealed an essential role of *fezl* in establishing proper subdivisions within the diencephalon: *fezl* is required to specify the prethalamus and to repress the fate of the ZLI starting at around the mid-somitogenesis stage. GOF analyses show that Fezl is capable of expanding the prethalamus at the expense of ZLI. These LOF and GOF data nicely complement each other and demonstrate that *fezl* has a crucial role in diencephalic regionalization.

In addition to affecting the diencephalon, Fezl GOF is also able to expand the telencephalon and hypothalamus at the expense of eyes and other fore- and/or mid-brain regions. However, the telencephalon and hypothalamus remain largely normal in *fezl* morphants. Although we cannot fully rule out the possibility that the

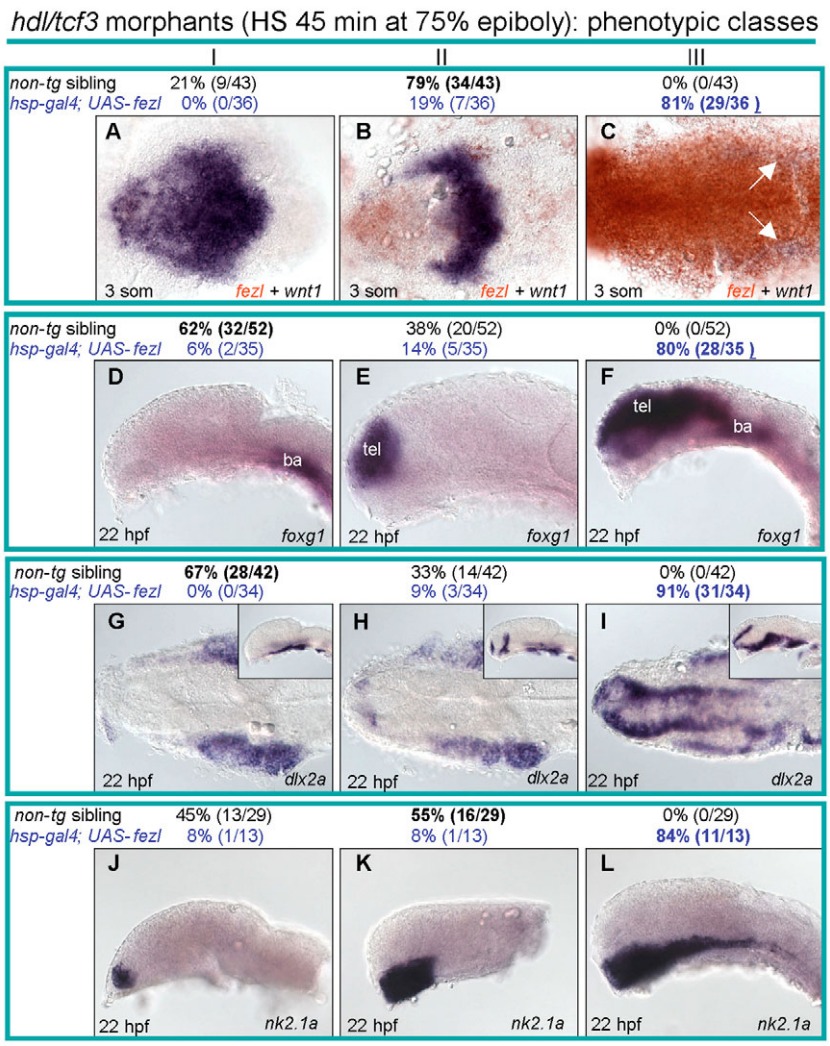

Fig. 7. *fezl* overexpression is sufficient to repress *wnt1* and to restore anterior forebrain gene expression in Headless and/or Tcf3-deficient embryos. (**A-L**) Analysis of gene expression. The *hdl/tcf3* morpholino was injected into one- to eight-cell-stage embryos derived from the *hsp-gal4/+*×*uas-fezl* cross. These *hdl/tcf3* morphants were subjected to heat shock at ~75% epiboly, analyzed for forebrain gene expression at ~28 hpf with various markers (as specified in each panel) and subsequently genotyped for the presence or absence of the transgenes. In general, three classes of phenotypes were observed for each marker, and their percentage distributions in the non-transgenic heat-shocked *hdl/tcf3* morphants versus in the double transgenic heat-shocked *hdl/tcf3* morphants are indicated above each panel. A-C,G-I are dorsal views and D-F,J-L are lateral views. Insets in G-I show lateral views of the same embryos. Arrow in C represents residual *wnt1*-expressing cells. tel, telencephalon; ba, branchial arches.

GOF phenotypes observed for the telencephalon and hypothalamus are 'artifacts' due to overexpression of *fez1*, several lines of evidence suggest that it is not the case. First, such GOF phenotypes were not observed in embryos that were heat-shocked at later stages, suggesting that these phenotypes are not simply due to an overproduction of Fezl. Second, whereas anterior-posterior patterning is disturbed in *fezl* GOF embryos, other axial development (e.g. dorsoventral patterning) appears largely normal. Finally, endogenous *fezl* expression was detected strongly in both the telencephalon and hypothalamus during the early development of these embryos. Therefore, one plausible explanation for the strong GOF but weak LOF phenotypes in the telencephalon and hypothalamus is that other forebrain-expressed factors may compensate for the loss of Fezl in these brain regions. One such factor might be Six3, which is essential for murine forebrain development (Lagutin et al., 2003) and medaka fish eye formation (Carl et al., 2002; Del Bene et al., 2004), and has also been shown to regulate forebrain development in zebrafish (Ando et al., 2005; Kobayashi et al., 1998). Another compensating factor may be *lhx5*, which was recently shown to promote forebrain development via the transcriptional activation of secreted Wnt antagonists (Peng and Westerfield, 2006). Thus, it is possible that *fezl*, *six3* and *lhx5* together may ensure the proper establishment of multiple anterior neural subdivisions that include the telencephalon, eyes, prethalamus and hypothalamus.

Because *fezl* expression is not detectable in dopaminergic neurons (Levkowitz et al., 2003) a cell non-autonomous function of *fezl* has been previously proposed for their development. However, recent evidence favors the hypothesis that *fezl* is expressed in the dopaminergic progenitor cells to cell autonomously control their development (Jeong et al., 2006). Several lines of evidence suggest that *fezl* may also act cell autonomously in the specification of the prethalamus. First, *fezl* is expressed in the prethalamus. Second, *fezl* LOF leads to a deficit of prethalamus, whereas *fezl* GOF leads to an expansion of prethalamus. Third, *fezl* has previously been shown to cell autonomously induce the expression of *dlx2a*, a prethalamic marker (Yang et al., 2001). Whereas *fezl* is likely to act cell autonomously in the specification of the prethalamus, its requirement for repressing ZLI may be cell non-autonomous because *fezl* appears not detected in this region.

What are the mechanisms by which *fezl* regulates diencephalic regionalization? One possible avenue is through repressing the target genes of the wnt and/or β-catenin signaling pathway, which has been shown to repress the anterior, and promote the posterior, neural fate. Our LOF analysis shows an expansion of *irx3a*, a gene induced by *wnt* signaling (Braun et al., 2003). Our GOF analysis demonstrates the capability of *fezl* to repress *wnt1* and *pax2a*, which is followed by the loss of two important organizers – the ZLI and MHB – which may be the cause of the GOF phenotypes. Moreover, the rescue of the *headless/tcf3*-deficient embryos by *fezl* overexpression further

substantiates the ability of Fezl to repress *wnt* signaling. The largely unaffected *wnt* expression in the *fezl* morphants (see Fig. S2E-J in the supplementary material) may be due to genetic compensation, possibly provided by *six3* or *lhx5*. Alternatively, the interaction between *fezl*- and wnt-signaling may not be at the level of direct transcriptional regulation of the wnt family of genes. Taken together and consistent with a suggested requirement of inhibiting *wnt* activity for the development of the prethalamus (Braun et al., 2003; Kiecker and Niehrs, 2001; Lagutin et al., 2003), our results provide strong evidence that such repression of *wnt* activity can be achieved by *fezl*. Finally, it is worth pointing out that our data are consistent with the possibility that *fezl* may also play a role in directly promoting the prethalamic fate independent from repressing *wnt* activity.

A clear understanding of the biochemical mechanisms underlying *fezl* function requires the future identification of its direct target genes. Moreover, it is also of great interest to know mechanistically how *fezl* expression is restricted to distinct anterior forebrain subdivisions. Consistent with the Wnt gradient hypothesis in forebrain regionalization (Wilson and Houart, 2004), *fezl* is inducible by Dkk1 (Hashimoto et al., 2000), again, making the Wnt pathway an attractive candidate in regulating *fezl* expression. Future studies will provide crucial insights into possible cross regulations between *fezl* and *wnt* signaling.

Our study uncovers an essential role of *fezl* in diencephalic patterning, prior to its function in neuronal subtype differentiation (Chen et al., 2005a; Chen et al., 2005b; Guo et al., 1999; Hirata et al., 2004; Jeong et al., 2006; Levkowitz et al., 2003; Molyneaux et al., 2005). Given its restricted expression in the vertebrate forebrain and a demonstrated role in regulating reward-associated behaviors (Lau et al., 2006), it would be interesting to test in the future whether the deregulation of Fezl may be involved in human neurological disorders that have a developmental origin.

While our manuscript was under review, it was reported that the mouse Fez and Fezl proteins together also play a crucial role in the establishment of the diencephalon divisions (Hirata et al., 2006a), suggesting an evolutionarily conserved role of the Fez- and Fezl-gene families. Interestingly, a notable difference exists between zebrafish and mice: whereas our LOF and GOF analyses indicate a clear role of zebrafish *fezl* in promoting the prethalamus and in repressing the ZLI, *fez*$^{-}$*fezl*$^{-}$ double-mutant mouse embryos had a loss of both prethalamus and ZLI, but an expansion of caudal diencephalon (the thalamus and pretectum). However, similar to our GOF data in zebrafish, overexpression of mouse *fezl* or *fez* abolished *shh*-expressing ZLI. This difference in LOF phenotypes could be species-dependent. Alternatively, the zebrafish *fezl* morphant perhaps represents a weaker LOF of *fezl* (also with *fez* being intact), whereas the *fez*$^{-}$*fezl*$^{-}$ double-mutant mice are null conditions. Therefore, it is attractive to hypothesize that the levels of *fezl* and/or *fez* activity may determine distinct subdivisions along the rostrocaudal axis. Future experiments are necessary to test this hypothesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02705/DC1

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