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Overlapping and distinct functions provided by *fgf17*, a new zebrafish member of the Fgf8/17/18 subgroup of Fgfs

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Abstract

Members of the fibroblast growth factor (Fgf) family are important signaling molecules in several inductive and patterning processes, and act as brain organizer-derived signals during formation of the early vertebrate nervous system. We isolated a new member of the Fgf8/17/18 subgroup of Fgfs from the zebrafish, and studied its expression and function during somitogenesis, optic stalk and midbrain-hindbrain boundary (MHB) development. In spite of a slightly higher aminoacid similarity to Fgf8, expression analysis and mapping to a chromosome stretch that is syntenic with mammalian chromosomes shows that this gene is orthologous to mammalian *Fgf17*. These data provide a further example of conserved chromosomal organization between zebrafish and mammalian genomes. Using an mRNA injection assay, we show that *fgf17* can act similar to *fgf8* during gastrulation, when *fgf17* is not normally expressed. Direct comparison of the expression patterns of *fgf17* and *fgf8* suggest however a possible cooperation of these Fgfs at later stages in several tissues requiring Fgf signaling. Analysis of zebrafish MHB mutants demonstrates a gene-dosage dependent requirement of *fgf17* expression for the *no isthmus// pax2.1* gene, showing that *no isthmus/pax2.1* functions upstream of *fgf17* at the MHB in a haplo-insufficient manner, similar to what has been reported for mammalian pax2 mutants. In contrast, only maintenance of *fgf17* expression is disturbed at the MHB of *acerebellar/fgf8* mutants. Consistent with a requirement for *fgf8* function, implantation of FGF8-soaked beads induces *fgf17* expression, and expression is upregulated in *aussicht* mutants, which display upregulation of the Fgf8 signaling pathway. Taken together, our results argue that Fgf8 and Fgf17 act as hierarchically organized signaling molecules during development of the MHB organizer and possibly other organizers in the developing nervous system.

Keywords: Neurogenesis; *Fgf17*; *Fgf8*; *Fgf18*; Midbrain; Hindbrain; Midbrain-hindbrain boundary; Optic stalk; Somites; Isthmus; Zebrafish; *Danio rerio*

1. Introduction

Subdivision of the vertebrate neural plate is regulated by the activity of distinct cell populations with organizing character (Marin and Puelles, 1994; Lumsden and Krumlauf, 1996; Houart et al., 1998). One such cell population is located in the boundary territory between midbrain and hindbrain (MHB), which at later stages forms the isthmus rhombencephali (Alvarado-Mallart, 1993; Wassef et al., 1993; Nakamura et al., 1994; Marin and Puelles, 1994; Bally-Cuif and Wassef, 1995; Joyner, 1996; Puelles et al., 1996; Wassef and Joyner, 1997; Brand, 1998). Transplantation experiments in the chick embryo suggested that the MHB functions as an organizing center which influences the fate of the surrounding neural tube cells. When MHB tissue is grafted into caudal forebrain or hindbrain, ectopic midbrain or cerebellar structures are induced, respectively (Gardner and Barald, 1991; Martinez et al., 1991, 1995; Bally-Cuif et al., 1992; Marin and Puelles, 1994).

Several molecules are expressed at the MHB that are important for the activity of the MHB organizer. For several transcription factors, such as En1 and En2 (Wurst et al., 1994; Millen et al., 1994), Pax2 (Torres et al., 1995; Brand et al., 1996; Favor et al., 1996; Lun and Brand, 1998), Pax5 (Urbanek et al., 1994), Otx1 and Otx2 (Ang et al., 1996; Acampora et al., 1997) and Gbx2 (Wassarman et al., 1997), a requirement during development and/or function of the MHB has been demonstrated. Among the secreted factors, the vertebrate homolog of *Drosophila* Wingless, Wnt1 (Thomas and Capecchi, 1990; McMahon et al., 1992) and Fgf8, a member of the fibroblast growth factor family, are required for MHB development in mice

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(Meyers et al., 1998) and zebrafish (Reifers et al., 1998; Picker et al., 1999).

To better understand the function of the MHB organizer, the analysis of loss-of-function mutants has been of great value. In zebrafish, lethal mutations in four genes affect development of the MHB. Homozygous *acerebellar* (*ace*; Brand et al., 1996) and *spiel-ohne-grenzen* (*spg*; Schier et al., 1996) embryos lack the MHB and the cerebellum, but retain a midbrain. *acerebellar* is a mutation in the zebrafish *fgf8* gene (Reifers et al., 1998). In *aussicht* (*aus*; Heisenberg et al., 1999) mutant embryos *fgf8* is upregulated in most of its expression sites, suggesting that *aus* may be involved in feedback-regulation of *fgf8* transcription. Embryos homozygous for *no isthmus* (*noi*) lack the MHB and cerebellum, as well as the midbrain, and *noi* mutations inactivate the *pax2.1* transcription factor (Brand et al., 1996; Lun and Brand, 1998); the genes affected by *spg* and *aus* are not yet known.

Fibroblast growth factors (Fgfs) in general serve as signaling molecules during development. For instance, injection of dominant negative Fgf receptor constructs in *Xenopus* and zebrafish leads to posterior truncation, demonstrating that Fgf signaling is required during gastrulation and mesoderm development (Griffin et al., 1995; Kroll and Amaya, 1996). The Fgf family consists of at least 19 members in vertebrates (Xu et al., 1999; Nishimura et al., 1999; Ornitz, 2000; and references therein). Fgfs signal through four receptor-tyrosine kinases (Fgfr1 to −4) through the MAP-Kinase pathway (Basilico and Moscatelli, 1992). Among the known members of the Fgf family, *Fgf3*, *Fgf8*, *Fgf17* and *Fgf18* are expressed at the MHB in different species (Tannahill et al., 1992; Mahmood et al., 1996; Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Fürthauer et al., 1997; Reifers et al., 1998; Hoshikawa et al., 1998; Xu et al., 1999; Ohbayashi et al., 1998). An essential function of Fgf8 in mediating the organizer activity of the MHB could be demonstrated through gain- and loss-of-function experiments (Crossley et al., 1996; Meyers et al., 1998; Reifers et al., 1998; Picker et al., 1999). In addition, Fgf8 signaling has been implicated in the function of a second organizing cell population involved in forebrain patterning (Shimamura and Rubenstein, 1997; Houart et al., 1998; Shanmugalingam et al., 2000), and in heart and otic placode induction and development (Reifers et al., 2000; Léger and Brand, in preparation). In zebrafish, only two members of the Fgf family, Fgf3 and Fgf8, have been described so far (Kiefer et al., 1996; Fürthauer et al., 1997; Reifers et al., 1998). Both *fgfs* are expressed at the MHB from gastrulation stages onwards (Fürthauer et al., 1997; Reifers et al., 1998; Reifers and Brand, unpublished data).

Here we report the isolation and characterization of a new member of the zebrafish Fgf family, *fgf17*, which is expressed in a partially overlapping pattern compared to *fgf8*. The expression pattern of *fgf17* at the MHB, the optic stalks and the somites, as well as gain-of-function experiments suggest important functions for Fgf17. The analysis of several zebrafish MHB mutants and implantations of beads coated with Fgf8 protein reveal that Fgf17 probably serves an important function within the genetic hierarchy of the MHB organizer.

2. Results

2.1. Cloning and identification of zebrafish fibroblast growth factor 17

Using the mouse *Fgf8* cDNA as a probe, we have isolated a new zebrafish *fgf* from a genomic zebrafish cosmid library. Screening of a cDNA library with the cosmid clone as probe resulted in a 2.4 kb cDNA encoding a new *fgf* gene. The amino acid sequence of this gene is 70% identical to zebrafish *fgf8*, 66% identical to mouse *fgf8*, 54% identical to mouse *fgf17* and 50% identical to mouse *fgf18* (Fig. 1A). Phylogenetic sequence comparison indicates that the new *fgf* gene belongs to the *fgf8/17/18* subgroup of *fgfs* (Fig. 1A,C). A N-linked glycosylation site at position 140 is shared by all members of the fgf8/17/18 subgroup. A second N-linked glycosylation site at position 34 is characteristic for *fgf8*, but is lacking in mouse *fgf17* and *fgf18* and in the newly identified zebrafish *fgf*.

To determine whether the new gene is a duplicated *fgf8,* or a zebrafish ortholog of other members of the *fgf8/fgf17/fgf18* subgroup, we have mapped its genetic position in the genome (Fig. 1D). *fgf8* maps to linkage group 13 to an area that is syntenic with mammalian chromosomes, in close proximity to *pax2.1*. The duplicated *pax2.2* gene maps to linkage group 12 of the zebrafish genome (J.H. Postlethwait, personal communication), and a duplicated *fgf8* gene might therefore map close to *pax2.2* on linkage group 12. However, the newly identified *fgf* maps to linkage group 1 to an area which contains several genes with conserved synteny with mouse chromosome 14. Within the syntenic stretch, the new *fgf* occupies the position of murine *fgf17*. Together with the expression analysis (see below), these data show that the new *fgf* is the zebrafish ortholog of mammalian *fgf17*, and will be referred to as zebrafish *fgf17*.

2.2. Expression pattern of zebrafish fibroblast growth factor 17

To study possible functions of *fgf17*, we examined expression in wild type embryos using whole-mount in situ hybridization (Fig. 2). Unlike *fgf8*, *fgf17* is not expressed during gastrulation (not shown). Expression becomes first detectable at the 8-somite stage at the MHB in a region coincident with *fgf8*, and in the anterior somites (Fig. 2A–E). Expression at the MHB is broad ventrally and is excluded from the dorsal-most part. From the 14-somite stage onwards, *fgf17* is expressed in the optic stalks (Fig. 2F). Expression in the somites becomes confined to the anteromedial margin of the maturing somite (Figs. 2G and

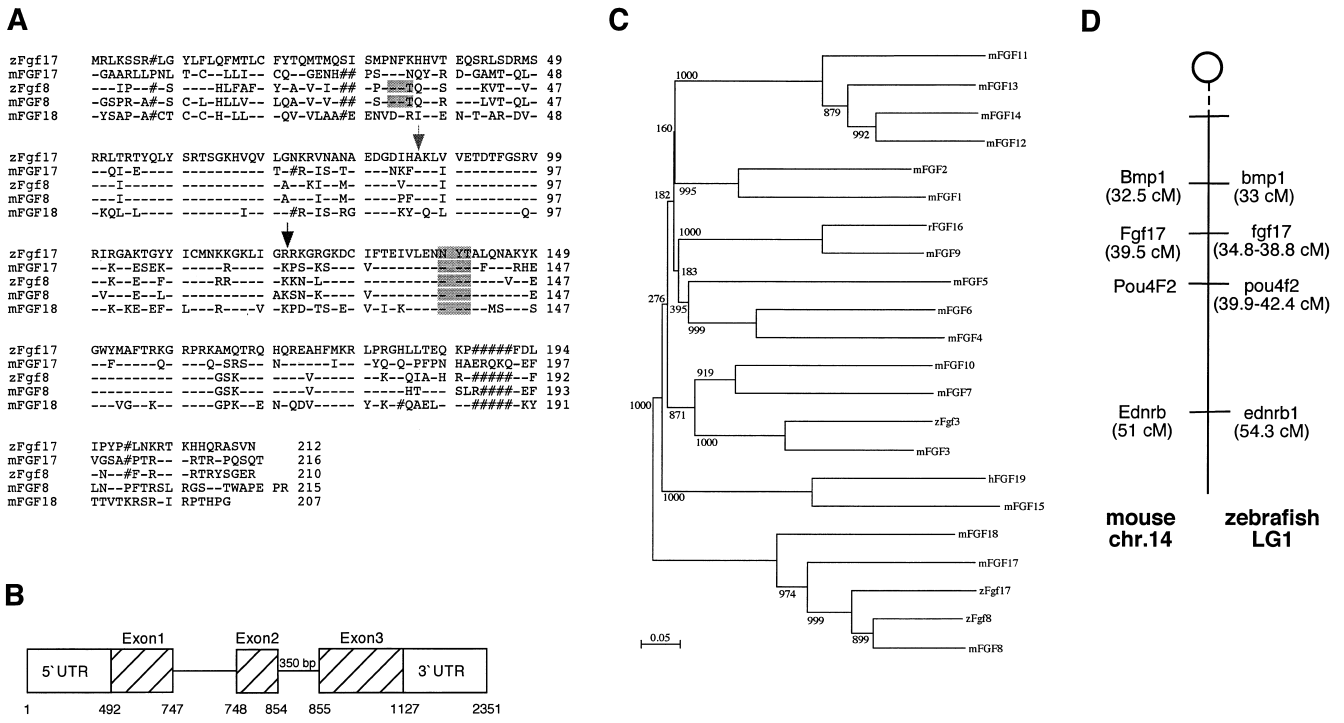

Fig. 1. (A) Sequence comparison between the predicted amino acid sequences of zebrafish Fgf17, mouse FGF17, zebrafish Fgf8, mouse FGF8 and mouse FGF18 proteins. Horizontal bars indicate identical residues, # indicate introduced gaps; arrows mark exon boundaries; N-linked consensus sites are shaded. (B) Genomic structure of the *fgf17* gene. Exons are shown as boxes, introns are shown as lines; hatched boxes represent the open reading frame. (C) Phylogenetic tree comparing zebrafish Fgf17 with the other known FGFs. Numbers indicate boot strap values. (D) Syntenic relationship between zebrafish linkage group 1 and mouse chromosome 14.

3E). At 24 h, expression at the MHB is confined to the ventricular zone and excluded from the floorplate and the dorsal-most aspect of the neural tube (Fig. 2I,J,P). Expression in the somites is lost after somitogenesis (Fig. 2M,N). In addition, *fgf17* is expressed in the otic vesicle at 24 h, in an area where the anterior macula will form (Fig. 2L). At 30 h, expression starts in the hyoid, and at 36 h in the dorsal diencephalon close to the forming epiphysis (Fig. 2N,O).

2.3. Comparative analysis of fgf17 and fgf8 expression patterns

Because *fgf17* expression resembles the pattern of *fgf8* expression (see Reifers et al., 1998), we have directly compared the expression of these two genes by double in situ hybridization. The domains at the MHB are broadly overlapping, but only *fgf8* is expressed in the dorso-anterior forebrain (Fig. 3A–D). While *fgf8* is expressed throughout the anterior margin of the maturing somites, *fgf17* is expressed only in the anteromedial subpopulation (Fig. 3E,F). Similarly, *fgf17* expression can only be detected in a medial subpopulation of *fgf8* expressing cells in the optic stalk, but not in facial ectoderm or nasal placode, and only weakly in the dorsal diencephalon (Fig. 3G–L). In summary, the expression domains of *fgf17* and *fgf8* largely overlap, with *fgf17* being expressed in a subpopulation of *fgf8*-positive cells, with the exception of the forebrain primordium where there is only *fgf8* expression.

2.4. fgf17 can posteriorize and dorsalize zebrafish embryos

To analyze potential functions of Fgf17 in the developing zebrafish embryo, we performed gain-of-function experiments. Unlike *fgf8* (Fürthauer et al., 1997; Reifers et al., 1998), injection of *fgf17* RNA into wild type embryos at the one- to two- cell-stage results in a dose-dependent anterior shift at tailbud stage of *pax2.1* and *krox20* as markers for midbrain and rhombomeres 3 and 5, respectively (Fig. 4, Table 1A); control injections with lacZ RNA had no effect (not shown). Similar to *fgf8*, *fgf17* misexpression causes severe expansion of both markers to ventral levels, which often encircle the embryo (Fig. 4). This phenotype is indicative of dorsalization, as is more obvious during early somitogenesis, as monitored by in situ hybridization with *myoD* (Fig. 4, Table 1B). We conclude that like *fgf8*, *fgf17* can exert a dorsalizing effect on the zebrafish gastrula; in addition, fgf17 also causes posteriorization. Although these data show that fgf17 has a strong potency to influence gastrula patterning, the observed effects cannot reflect an endogenous function of *fgf17* in the zebrafish embryo, since *fgf17* is not expressed during gastrulation. More likely, *fgf17* injection mimics the activity of other Fgf's (see Section 3). The early effects caused by *fgf17* RNA injection

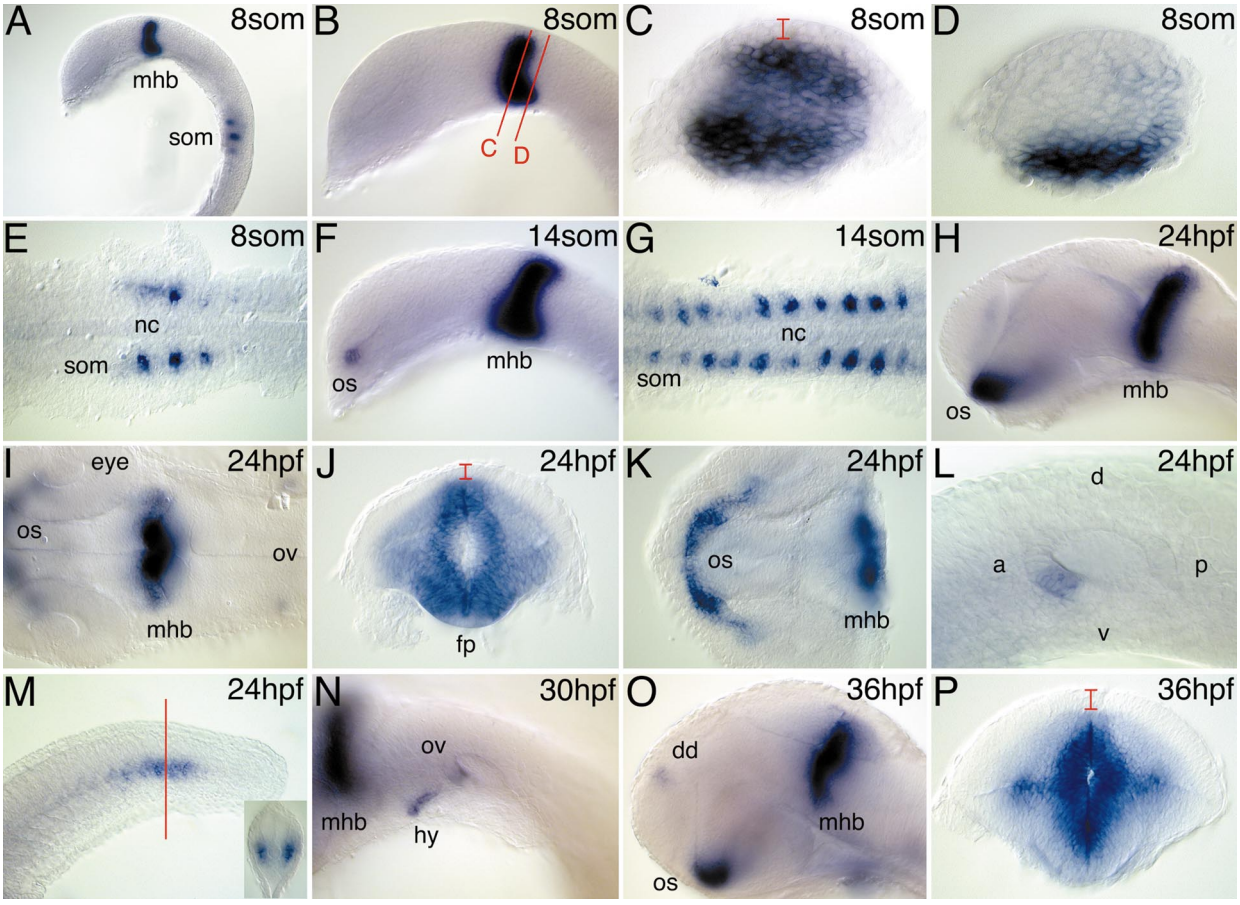

Fig. 2. Expression of *fgf17* in wild type embryos. (A) Onset of expression at the MHB and the somites at 8-somite stage. (B) Close-up of MHB expression. (C,D) Thick cross-sections through the MHB at indicated levels (B); note that *fgf17* is not expressed at the dorsal-most part of the MHB (bracket). (E) Dorsal view of expression in somites. (F) Onset of expression in the optic stalk at 14-somite stage. (G) Dorsal view of expression in somites at 14-somite stage. (H) At 24 hpf, *fgf17* is strongly expressed in the optic stalk and in a narrow band at the MHB. (I) Dorsal view of a 24 hpf embryo; expression is detected at the MHB, the optic stalks and the otic vesicles. (J) Thick cross-sections through the MHB at 24 hpf; expression is mainly confined to the ventricular zone; note that the dorsal-most part of the MHB (bracket) and the floorplate are devoid of *fgf17* expression. (K) Ventral view of expression in the optic stalks and the MHB at 24 hpf. (L) Onset of expression in the anteriorventral otic vesicle at 24 hpf. (M) Fading expression in somites close to the tailtip; inlet: section through tail at indicated level. (N) Onset of expression in the hyoid at 30 hpf. (O) At 36 hpf, *fgf17* can be detected in the dorsal diencephalon. (P) Thick cross-sections through the MHB at 36 hpf; expression is confined to the ventricular zone; note that *fgf17* is not expressed at the dorsal-most part of the MHB (bracket). All pictures show lateral views, except when indicated. Anterior is always to the left, except in sections. a, anterior; d, dorsal; dd, dorsal diencephalon; fp, floorplate; hy, hyoid; mhb, midbrain-hindbrain boundary; nc, notochord; os, optic stalk; ov, otic vesicle; p, posterior; som, somites; v, ventral.

so far precluded a gain-of-function analysis at later stages, e.g. in MHB development. Nevertheless, the difference in the response of the embryo towards *fgf8* and *fgf17* injection suggests that the encoded proteins have overlapping, but not identical properties.

2.5. *fgf17 expression in zebrafish MHB mutants*

Although *fgf17* orthologs have been isolated in several species, its position in the genetic hierarchy is unknown, partly because expression in mutant backgrounds has not yet been studied. We therefore studied *fgf17* expression in several zebrafish MHB and optic stalk mutants in a detailed time course (Fig. 5, Table 2). MHB expression of *fgf17* in *acerebellar* (*ace*) mutant embryos, in which *fgf8* is inactivated (Reifers et al., 1998), is almost absent at its onset and is lost by the 16-somite stage (Fig. 5B,F). *aussicht* (*aus*) is a dominant mutation causing an increase in *fgf8* RNA expression (Heisenberg et al., 1999); and in mutant embryos *fgf17* expression is initiated normally, but expands strongly at late somitogenesis (Fig. 5C,G). *spiel-ohne-grenzen* (*spg*) mutant embryos do not form a proper MHB (Schier et al., 1996) and fail to initiate expression of *fgf17* at the MHB, but recover the dorsal aspect of the expression domain at later stages (Fig. 5D,H). Importantly, *fgf17* is never expressed at the MHB in *no isthmus* (*noi*)/*pax2.1*-mutant embryos, which lack the MHB, cerebellum and midbrain at pharyngula stages (Fig. 5I–P). Heterozygous $noi^{+/-}$ embryos show a clear reduction in expression when compared to their wild type siblings at the 14- to 20-somite stage; thus, expression of *fgf17* depends on the copy number of the *pax2.1* gene (Fig. 5). These results show that *fgf17* is clearly dependent

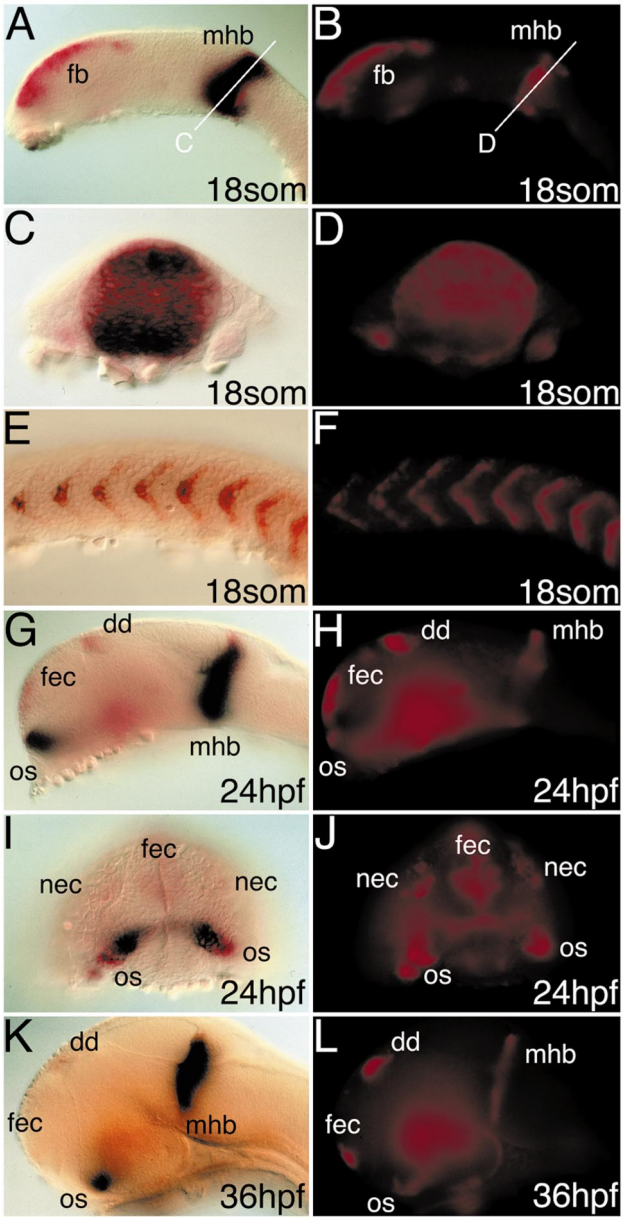

Fig. 3. Comparison of *fgf17* (blue) and *fgf8* (red, fluorescent) expression patterns by double ISH in wild type embryos at given stages. (A,B) Expression of *fgf17* and *fgf8* overlaps at the MHB, while in the forebrain only *fgf8* is expressed. (C,D) Thick cross-section through the MHB at level indicated in (A,B). (E,F) Expression of *fgf17* and *fgf8* in the somites; note that both genes are expressed at the anterior border of the somite, with *fgf17* being only expressed in the medial subpopulation of *fgf8*-positive cells. (G,H) At 24 hpf, *fgf17* and *fgf8* expression overlaps at the MHB and in the optic stalk, while only *fgf8* is expressed in the dorsal diencephalon and the facial ectoderm. (I,J) Frontal view on a brain at 24 hpf; *fgf17* is expressed only in a subpopulation of *fgf8*-positive cells in the optic stalks; the nasal and facial ectoderm exclusively express *fgf8*. (K,L) At 36 hpf, *fgf8* expression at the MHB reaches further dorsally and ventrally than *fgf17*. When not indicated, embryos are shown in lateral view with anterior to the left. (A,C,E,G,I,K) Bright-field images; (B,D,F,H,J,L) fluorescent images of the same embryo. dd, dorsal diencephalon; fb, forebrain; fec, facial ectoderm; mhb, midbrain-hindbrain boundary; nec, nasal ectoderm; os, optic stalk.

on *pax2.1* gene function, while *fgf8* is required for most, but not all of the initial fgf17 expression, and for maintaining *fgf17* expression at the MHB. The genes affected in *aussicht* and *spiel-ohne-grenzen* are similarly involved in maintaining *fgf17* expression at the MHB. In the optic stalk, *fgf17* expression is reduced in *ace*, *spg* and *noi*, but increased in *aus* mutants, suggesting a function of these genes in maintaining *fgf17* expression in the optic stalk.

2.6. Inhibition of Fgf signaling causes loss of fgf17 expression

The altered expression of *fgf17* in *ace*, *spg* and *aus* raised the possibility that *fgf8* might act upstream of *fgf17*. We therefore analyzed the expression in wild type embryos where Fgf signaling has been blocked pharmacologically by treatment with SU5402. SU5402 blocks Fgfr1 activity by binding to a region of the kinase domain that is identical in all four Fgfrs, and is thought to be a potent inhibitor of all Fgfr function (Mohammadi et al., 1997; Johnson and Williams, 1993). Treatment of wild type zebrafish embryos with SU5402 results in a phenocopy of several aspects of the *acerebellar* mutant phenotype (Reifers et al., 2000; Shanmugalingam et al., 2000). SU5402 treatment from tailbud stage onwards causes strong reduction of *fgf17* expression at the MHB (Fig. 5Q,R), similar to *acerebellar* mutants (Fig. 5A,B). This finding further supports the notion that *fgf17* expression depends on activity of Fgfs in general, and Fgf8 in particular. Moreover, it suggests that the early remnant expression of *fgf17* that is observed at the MHB in *acerebellar* mutants and in SU5402-inhibited embryos is independent of Fgf signaling.

2.7. fgf17 expression can be induced by an Fgf8 bead

To further test if Fgf8 itself could be an inducer of *fgf17*, we tested whether FGF8 protein-coated beads can induce expression of *fgf17* upon implantation. Ectopic *fgf17* expression was seen in two of five wild type embryos implanted with an Fgf8-bead at the 14-somite stage, in the telencephalon at the 20-somite stage (Fig. 6A,B) and at 33 h in the ventral midbrain (Fig. 6C). To examine whether Fgf8

Table 1
Summary of *fgf17* misexpression

Fgf17 RNA injection	25 pg	12.5 pg	2.5 pg	0.25 pg
(A)				
No effect	9%	8%	8%	80%
Pax2.1 still present	44%	53%	61%	15%
Pax2.1 absent	27%	21%	25%	0%
Pax2.1 and krox20 absent	20%	18%	6%	5%
n embryos	45	38	51	54
(B)				
No effect	6%	3%	7%	60%
Broader somites	50%	19%	32%	24%
Messed up	44%	77%	61%	15%
n embryos	18	26	44	91

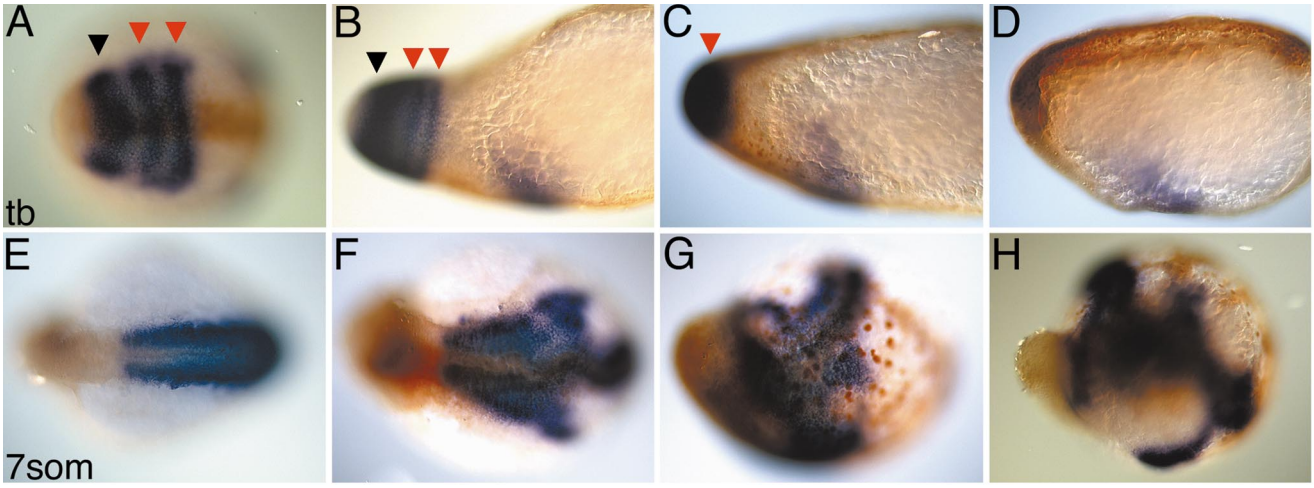

Fig. 4. Effect of *fgf17* misexpression on zebrafish gastrula. The MHB primordium is visualized with *pax2.1*, rhombomere 3 and 5 by *krox20* and adaxial and somitic mesoderm by *myoD*; location of the *lacZ* co-injected cells with an antibody to beta-gal (brown). For quantification of the phenotypes, see Table 1. (A–D) Misexpression of *fgf17* in wild type embryos by RNA injection results in posteriorization. (A) *pax2.1* (black arrowhead) and *krox20* (red arrowhead) expression in uninjected or control-lacZ injected embryos is restricted to the dorsal side (control; dorsal view). (B–D) Dose-dependent anterior shift of these markers in an *fgf17* injected embryos; note that expression of the normally dorsal marker expression now encircles the embryo, in the lateral view in (B) (indicative of dorsalization at this stage; compare also to Reifers et al., 1998), and that with increasing dose expression is shifted anteriorly. (E–H) Misexpression of *fgf17* in wild type embryos by RNA injection results in dorsalization. (E) *myoD* expression in unaffected embryo (control) labels the segmental plate mesoderm. (F–H) Dose-dependent broadening of somites and mislocation of mesoderm structures in *fgf17* injected embryos reflecting the severe dorsalization of the embryos. Embryos in (A,E–H) are shown in dorsal views, embryos in (B–D) are shown in lateral views, anterior to the left in all embryos, stages as indicated.

protein can induce *fgf17* expression at the MHB, we implanted Fgf8 beads into the MHB territory of homozygous *ace* mutants at the 14-somite stage, which normally lack *fgf17* expression. At the 20-somite stage, *fgf17* induction is observed in two of four *ace* embryos receiving an FGF8 bead (Fig. 6B); in the two non-induced embryos the bead was not located at the MHB. Control implantations of FGF8-soaked beads into the MHB territory of *ace* embryos lead to morphological rescue at 33 hpf, and induction of *sprouty4*, a target gene for Fgf8 signaling (Fig. 6D; M. Brand, unpublished observations). In all rescued cases the *ace* embryos showed a unilateral morphological constriction at the MHB close to the bead (Fig. 6D, arrow). Implantation of PBS beads had no effect. In summary, Fgf8 is not only necessary but also sufficient to induce expression of *fgf17* at the MHB during the time of normal expression, and the induction correlates with the morphological rescue of the MHB in *ace* embryos.

3. Discussion

Our analysis demonstrates a dosage-dependent requirement for *fgf17* for *noi/pax2.1* activity in the MHB organizer and the optic stalk, and a requirement for *acerebellar/fgf8* in correct onset and maintenance of the expression of *fgf17* during MHB organizer development. Our gain-of-function experiments support both redundant and distinct functions of Fgf17 in comparison to Fgf8. In addition, the chromosomal location of Fgf17 provides a striking example for long-range conservation of genome organization between zebrafish and mammals.

3.1. Cloning and expression of zebrafish fgf17

Within the Fgf family, the newly isolated zebrafish *fgf* fits into the subfamily which includes *fgf8* and mammalian *Fgf17* and *Fgf18*. Both in terms of sequence and expression patterns, this subgroup is distinct from other *Fgf* subgroups, including for instance *Fgf7* and *Fgf10*, or *Fgf4* and *Fgf6* (Fig. 1B; Nishimura et al., 1999; Xu et al., 1999). Although analysis of the amino acid identity suggests a slightly closer relationship to zebrafish *Fgf8* than to mammalian *Fgf17* (66 versus 54%), comparison of expression patterns and the conservation of chromosomal synteny show that the new zebrafish *fgf* is the ortholog of mammalian *Fgf17*, and will thus be designated *fgf17*. This situation most likely reflects the common ancestry of these genes, with the ancestral gene of *Fgf8* and *Fgf17* being more *Fgf8* like, whereas the mouse *Fgf17* has diverged more.

The overlapping expression pattern of *fgf17* and *fgf8* suggests redundant functions of these two Fgfs. In agreement with a functional redundancy of Fgf17 and Fgf8, it has been demonstrated, using a tissue-culture proliferation assay, that both Fgfs can activate the same Fgf receptors (Fgfr 2c, 3c and 4; Ornitz et al., 1996; Greene et al., 1998; Xu et al., 1999, 2000). Despite the broadly similar expression patterns, clear differences can be observed. Several structures express *fgf8* but completely lack expression of *fgf17* (apical ectodermal ridge, facial and nasal ectoderm,

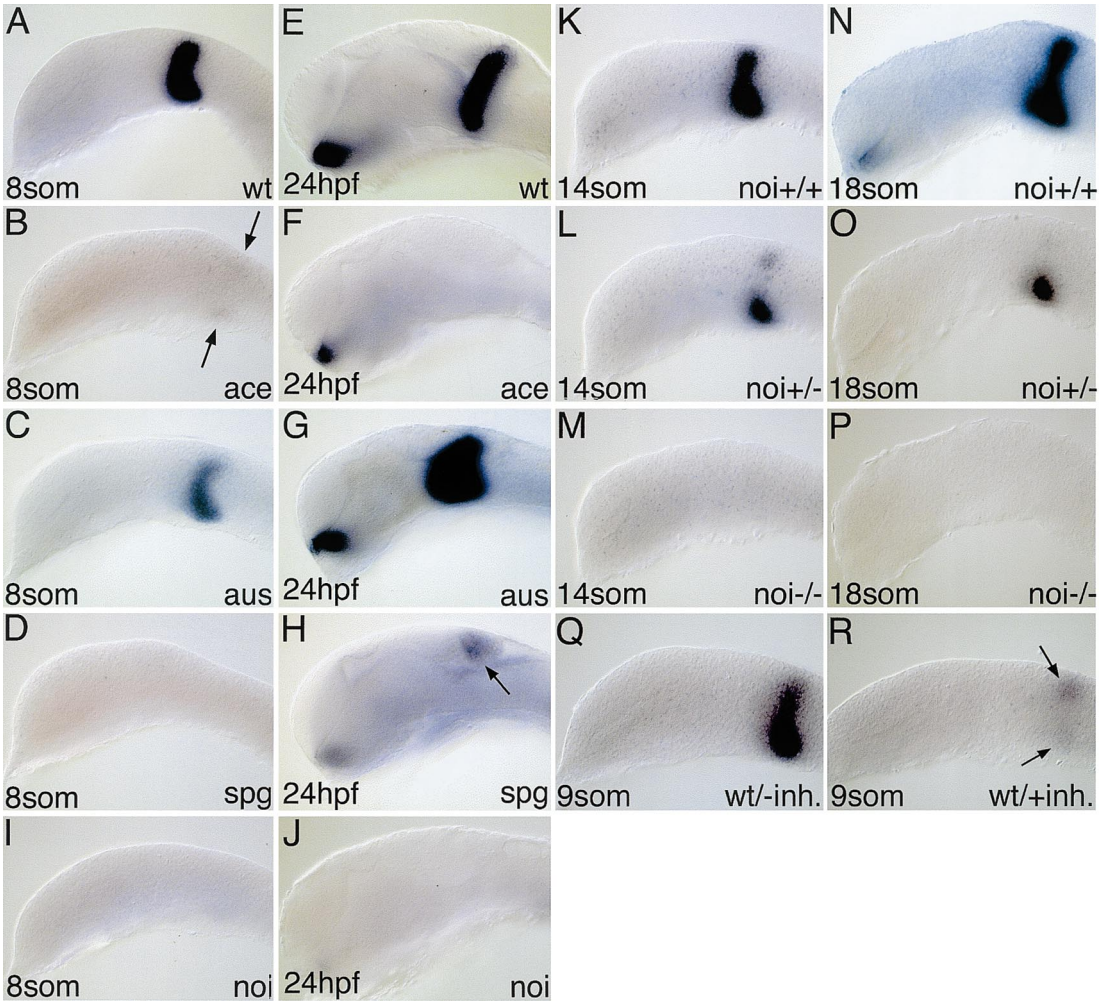

Fig. 5. Expression of *fgf17* in MHB mutants. Lateral views of dissected brain primordia at indicated stages. (A,E) Expression in wild type, (B,E) *acerebellar*, (C,G) *aussicht*, (D,H) *spiel-ohne-grenzen* and (I,J) *no isthmus* embryos. Note the remnant expression in *ace* at early stage (B, arrows) and in *spg* at late stage (H, arrow). (K–P) Haploinsufficiency for *fgf17* in *noi*. (K,N) Expression in wild type, (L,O) *noi* heterozygous and (M,P) *noi* homozygous embryos. (Q,R) Onset of *fgf17* expression is not completely dependent on Fgf signaling. (R) Wild type embryo continuously treated after gastrulation with Fgf inhibitor, (Q) untreated wild type embryo (control). Note remnant expression at MHB (arrows).

retina; Figs. 2 and 3, compare to Reifers et al., 1998). In addition, *fgf8* expression is detectable much earlier in many structures like the MHB, the dorsal diencephalon and the somites (Fig. 2). Similarly, murine FGF8 expression dominates during early developmental stages of the midbrain and the cerebellum (Xu et al., 2000). These differences in expression pattern suggest that Fgf17 and Fgf8 have acquired unique functions which seem to be conserved throughout vertebrate evolution (Xu et al., 1999, 2000).

3.2. Possible functions of zebrafish fgf17 in MHB development

The RNA misexpression experiments resulted in a severe dorsalization of the embryo and in an anterior shift of midbrain and hindbrain markers. The early effects caused by *fgf17* RNA injection so far precluded a gain-of-function analysis at later stages, e.g. in MHB development. Interestingly, Fgf17 dorsalizes the zebrafish gastrula more strongly than Fgf8, even at low RNA concentrations (Fürthauer et al., 1997; Reifers et al., 1998). In a mouse tissue culture proliferation assay, Fgf17 and Fgf8 can activate the same Fgf receptor profile (Ornitz et al., 1996; Greene et al., 1998; Xu et al., 1999, 2000). The different effects of Fgf8 and Fgf17 in gastrulation might therefore reflect a higher affinity

Table 2
fgf17 expression at the MHB in indicated mutants in a detailed time course[a]

	8 som	10 som	12 som	14 som	18 som	20 som	24 hpf
wt	+	+	+	+	+	+	+
ace	+/−	+/−	+/−	+/−	−	−	−
aus	+	+	+	+	+	++	+++
spg	−	−	−	−	−	−	+/−
noi	−	−	−	−	−	−	−

[a] +, normal; +/−, reduced; −, absent; ++/+++, upregulated.

of Fgf17 to the Fgf receptors that mediate Fgf signaling during gastrulation. Alternatively, the receptor specificity in vivo could differ from the tissue culture situation, or could differ between mouse and zebrafish.

Although the injection assay reveals a potential biological activity for Fgf17, this cannot be its endogenous function, since endogenous Fgf17 is not normally present in the gastrula. *fgf17* injection probably mimics the activity of other Fgfs that are present, such as *fgf8* and *fgf3* (Fürthauer et al., 1997; Reifers et al., 1998; Lun et al., unpublished observations). The anterior shift of midbrain and hindbrain markers by *fgf17* RNA injections is consistent with a role for Fgf signals in posteriorizing neural plate tissue (Lamb and Harland, 1995; Cox and Hemmati Brivanlou, 1995). The posteriorizing effect of *fgf17* is reminiscent of the influence that Fgf3 exhibits on the neural plate, and *fgf3* is expressed during gastrulation in zebrafish embryos (Lun et al., unpublished observations). However, in vitro Fgf3 activates a different subset of Fgf receptors (Ornitz et al., 1996), suggesting that another Fgf might act as the endogenous posteriorizing signal.

The normal function of Fgf17 in zebrafish MHB development is not clear. Since *fgf17* is expressed during the maintenance phase of MHB development, it may ensure together with additional signals at the MHB the polarized expression of midbrain markers. Given the greater potency of *fgf17* versus *fgf8* in gastrulation, onset of *fgf17* at this stage may reflect a new signaling capacity of the MHB organizer. In agreement with this possibility, expression of many marker genes is restricted to the posterior part of the midbrain primordium specifically during the maintenance phase of MHB development, around the time of *fgf17* onset (Lun and Brand, 1998; Reifers et al., 1998). Our mapping of *fgf17* may facilitate isolation of a mutant in *fgf17*, thus helping to further elucidate the role of Fgf17 in zebrafish development by loss-of-function studies. A 'knock-out' of mouse *Fgf17* confirmed its importance in MHB development: mutants have a defective medial cerebellar anlage at late developmental stages, whereas early patterning events in the MHB region occur normally. Interestingly, this phenotype is increased in severity when the *fgf17* mutants have only one copy of *fgf8*, suggesting that *fgf8* and *fgf17* may cooperate in regulating cerebellar size (Xu et al., 2000).

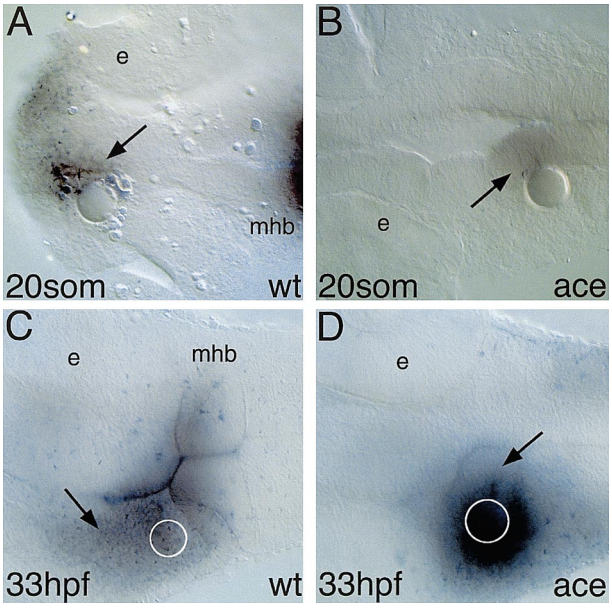

Fig. 6. FGF8 bead can induce *fgf17* expression in the zebrafish brain. (A) FGF8-soaked bead ectopically induces *fgf17* expression in the telencephalon of a wild type embryo (arrow). (B) Unilateral rescue of *fgf17* expression at the future MHB in *acerebellar* mutant embryo. (C) Ectopic expression of *fgf17* in the ventral midbrain of a wild type embryo after FGF8 bead implantation; the endogenous *fgf17* expression at the MHB is not visible in this focal plain. (D) Unilateral rescue of the MHB structure and reexpression of a target gene of Fgf8 signaling, *sprouty4*, in an *acerebellar* mutant embryo after FGF8 bead implantation. The arrow points to the rescued MHB fold. All pictures show dorsal views with anterior to the left, stages as indicated; circles in (C,D) indicate the position of the bead.

3.3. *fgf17* in the genetic hierarchy of the MHB organizer in zebrafish

An important finding in our study is that in $noi^{-/-}$ embryos *fgf17* expression is completely abolished at the MHB and the optic stalk, although the cells are present in the mutant at the time of onset of *fgf17* expression in these tissues (Lun and Brand, 1998; Pfeffer et al., 1998). *noi/pax2.1* is therefore a crucial upstream component in the pathway that activates *fgf17*, consistent with the colocalization of the expression domains at this stage (Lun and Brand, 1998). Interestingly, the expression of *fgf17* at the MHB and the optic stalk also depends on the gene dose of *pax2.1* at midsomitogenesis stages, since in $noi^{+/-}$ embryos *fgf17* expression is clearly reduced. Such haploinsufficiency has also been reported for the human *PAX2* gene, where heterozygous patients show optic nerve coloboma and vesicouretral reflux syndrome (Sanyanusin et al., 1995). Our observation suggests that *noi* heterozygous fish might provide useful models for these human diseases.

Our study supports a model of sequential or parallel action of *fgf8* and *fgf17* in MHB organizer development. Gain- and loss-of-function experiments argue in favor of an essential role of Fgf signaling in mediating the MHB organizer activity. FGF8-soaked beads can mimic the organizing activity of the isthmus and induce ectopic midbrain or cerebellar structures when implanted in the caudal forebrain of chick (Crossley et al., 1996; Martinez et al., 1999) or zebrafish (Picker, Reifers and Brand, unpublished) embryos. On the other hand, inactivation of the *Fgf8* gene in mice (Meyers et al., 1998) and zebrafish (Reifers et al., 1998) results in loss of the MHB organizer. Finally, an essential role has been demonstrated for Fgf8 signaling for growth and polarity of the midbrain (Picker et al., 1999; Lee et al., 1997). In zebrafish, Fgf8 is required for the maintenance of the MHB organizer as shown by the analysis of the

zebrafish *ace* mutant (Reifers et al., 1998). *fgf17* expression starts around the onset of the maintenance phase, consistent with a possible function in the MHB organizer. The gradual disappearance of *fgf17* expression at the MHB in *ace* mutant embryos is reminiscent of other MHB marker genes (Reifers et al., 1998), suggesting that *fgf17* is not only or not directly dependent on *fgf8* gene function. In accordance, the inhibitor experiments demonstrate that at least the onset of *fgf17* expression at the MHB is partially independent of Fgf signaling. Expression of *pax2.1* and *eng2* are strongly reduced and subsequently lost in the maintenance phase in *ace* mutant embryos. Given the absolute requirement for *pax2.1*, reduction of *fgf17* may be the indirect consequence of the reduction of *pax2.1* in *ace* mutants. Furthermore, the reexpression of *fgf17* at the MHB in *ace* mutant embryos at 20-somite stage after implantation of an FGF8-soaked bead supports an indirect mechanism, since FGF8 beads can induce ectopic *En* and *Pax2* expression in the developing neural plate (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). Similarly, upregulation of *fgf17* expression at the MHB in *aus* mutant embryos is in agreement with this view, since *fgf8* and *pax2.1*expression is upregulated at the MHB in *aus*, resulting in a broader expression of *eng* genes (Heisenberg et al., 1999). Future studies will determine whether the induction of *fgf17* expression is a direct effect of Pax2.1 activation or occurs indirectly through Eng activation.

4. Experimental procedures

Zebrafish were raised and kept under standard laboratory conditions at about 27°C (Westerfield, 1994; Brand and Granato, 2000). Mutant carriers were identified by random intercrosses. To obtain embryos showing the mutant phenotype, two heterozygous carriers for a mutation were crossed to one another. Typically, the eggs were spawned synchronously at dawn of the next morning, and embryos were collected, sorted, observed and fixed at different times of development at 28.5°C. In addition, morphological features were used to determine the stage of the embryos, as described by Kimmel et al. (1995). In some cases, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. Isolation and characterization of *acerebellar* (*ace*^{ti282a}), *no isthmus* (*noi*^{tu29a}), *spiel-ohne-grenzen* (*spg*^{m216}) and *aussicht* (*aus*^{t294}) are described in Brand et al. (1996); Reifers et al. (1998); Brand et al. (1996); Lun and Brand (1998); Schier et al. (1996); Heisenberg et al. (1999), respectively.

4.1. Isolation of fgf17 cDNA

A genomic zebrafish cosmid library was screened with the mouse *Fgf8* cDNA resulting in a 2 kb fragment which was used to screen a zebrafish cDNA library (_ZAPII, 9-16 hpf). The obtained phage contained a cDNA of 2351 bp (Accession number xxx). Both clones were sequenced and analyzed using the Analyze (MacMolly®Tetra, Version 1.2.3, Soft Gene GmbH) and the ClustalX software. The cDNA contained an open reading frame of 636 bp encoding a protein of 212 amino acids with a calculated molecular weight of 24 978 kDa. The exon/intron structure was determined by comparing the genomic clone with the cDNA clone. Exon/intron junctions have been confirmed by sequencing.

4.2. Mapping of fgf17 on the zebrafish genome

We mapped zebrafish *fgf17* on the Goodfellow T51 radiation hybrid panel (Research Genetics) using the following primer pair: CGA CCC AGG AAA GCC ATG CAG ACC C (5′primer) and TCA TTG TCC CAG AAC ATA CTG TTC C (3′primer). The 5′primer binds in the open reading frame, while the 3′primer is located in the 3′UTR of the gene. The PCR gives a fragment of 329 bp. PCR, gel electrophoresis and scoring was done as described in Geisler et al. (1999). PCR reactions were run in duplicates and uncertain results were confirmed in a third run.

4.3. Whole-mount in situ hybridization

In situ hybridizations were done as described by Reifers et al. (1998). Probes and wild type expression patterns are described: *fgf8* (Fürthauer et al., 1997; Reifers et al., 1998), *krox20* (Oxtoby and Jowett, 1993), *myoD* (Weinberg et al., 1996) and *pax2.1* (Krauss et al., 1991).

4.4. RNA injections

RNA injections were done as described by Reifers et al. (2000). During injection, RNA was deposited into the cytoplasm of one- to two- cell-stage embryos. The embryos were fixed at tailbud stage or at seven- to ten-somite stage prior to in situ hybridization and antibody staining.

4.5. Bead implantations and inhibitor treatment

Bead implantations and inhibitor treatment were done as described by Reifers et al. (2000). FGF8b or PBS control beads were implanted in various brain regions of wild type and *acerebellar* mutant embryos at 13 somites, the embryos were fixed at 20 somites or 33 hpf prior to ISH. For pharmacological inhibition of Fgfr activity, wild type embryos were treated with SU5402 (Calbiochem; Mohammadi et al., 1997) at 8 μm in embryo medium at 28.5°C in the dark for the indicated time periods.

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