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FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals

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Fibroblast growth factors (FGFs) are signaling molecules of the isthmic organizer, which regulates development of the midbrain and cerebellum. Tissue-specific inactivation of one of the FGF receptor (FGFR) genes, *Fgfr1*, in the midbrain and rhombomere 1 of the hindbrain of mouse embryos results in deletion of the inferior colliculi in the posterior midbrain and vermis of the cerebellum. Analyses of both midbrain–hindbrain and midbrain-specific *Fgfr1* mutants suggest that after establishment of the isthmic organizer, FGFR1 is needed for continued response to the isthmic signals, and that it has direct functions on both sides of the organizer. In addition, FGFR1 appears to modify cell adhesion properties critical for maintaining a coherent organizing center. This may be achieved by regulating expression of specific cell-adhesion molecules at the midbrain–hindbrain border.

Keywords: cerebellum/Cre recombinase/development/FGF/isthmic organizer/midbrain

Introduction

Organizing centers established at the borders between developmental units are commonly used for tissue patterning during embryogenesis. One such organizing center is the isthmic organizer, which forms at the junction between developing midbrain and rhombomere 1 of the hindbrain. Transplantation studies with avian embryos have demonstrated that tissue containing the midbrain–hindbrain junction can induce cells in more anterior and posterior regions of the brain to adopt fates characteristic for midbrain and rhombomere 1 (Nakamura *et al*., 1988; Martinez *et al*., 1991, 1995). Under the control of the isthmic organizer the dorsal midbrain develops into superior and inferior colliculi, relaying visual and auditory stimuli, respectively. In turn, dorsal rhombomere 1 forms the cerebellum involved in processes such as motor coordination. Development of some of the ganglia in the ventral brainstem is also thought to be regulated by the isthmic organizer.

The molecular basis for the development and function of the isthmic organizer is beginning to be understood. The border of expression of *Otx2* and *Gbx2*, two homeodomain transcription factors, separates the cells of the future midbrain from the hindbrain and determines the position of the isthmic organizer (Millet *et al*., 1996, 1999; Broccoli *et al*., 1999). In addition to the early regionalization of the neurectoderm, signals from the mesoderm are required for the induction of genes, such as *En1* and *En2*, in the mid- and hindbrain region (Hemmati-Brivanlou *et al*., 1990; Ang and Rossant, 1993). Studies with avian embryos have suggested that fibroblast growth factors (FGFs), potentially FGF4 transiently expressed in the anterior notochord, are important for the induction of mid- and hindbrain-specific gene expression (Shamim *et al*., 1999). In addition, *Fgf8*, expressed in the cardiogenic mesoderm, has been suggested to play a role in the induction of the midbrain (Crossley *et al*., 1996). In addition to the *En* genes, the paired box transcription factor genes *Pax2* and *Pax5* are also activated early in the entire midbrain–hindbrain region.

Later in development, patterning and growth of the midbrain and hindbrain rely on the isthmic organizer, which forms in the neurectoderm at the *Otx2*/*Gbx2* border. An important signaling molecule of the isthmic organizer is FGF8. In the mouse embryo, *Fgf8* expression is activated after *Pax2* and *En1* in the entire rhombomere 1 of the hindbrain, and later restricted to a stripe in the most anterior hindbrain (Crossley and Martin, 1995). Both gain- and loss-of-function experiments have suggested that FGF8 is essential for the activity of the isthmic organizer. FGF8 containing beads can mimic the isthmic transplants in induction of midbrain and cerebellum in the diencephalon or cerebellum in the posterior hindbrain (Crossley *et al*., 1996; Martinez *et al*., 1999; Irving and Mason, 2000). In addition, zebrafish *Fgf8* mutants fail to maintain isthmic gene expression (Reifers *et al*., 1998), and a hypomorphic mutation in the mouse *Fgf8* gene causes midbrain and cerebellar defects (Meyers *et al*., 1998). Other FGF family members, such as *Fgf17* and *Fgf18*, are also expressed in the isthmic region and may contribute to organizer function (Maruoka *et al*., 1998; Xu *et al*., 2000).

Another important signaling molecule of the isthmic organizer is WNT1. Initially, *Wnt1* is detected in the entire midbrain, but later *Wnt1*-positive cells are found as a narrow stripe in the most posterior midbrain next to the *Fgf8*-expressing cells in the anterior hindbrain. FGF-containing beads can induce *Wnt1* expression, implicating *Wnt1* as one of the targets of FGF signaling (Crossley *et al*., 1996). On the other hand, *Fgf8* expression is down-regulated in the *Wnt1*

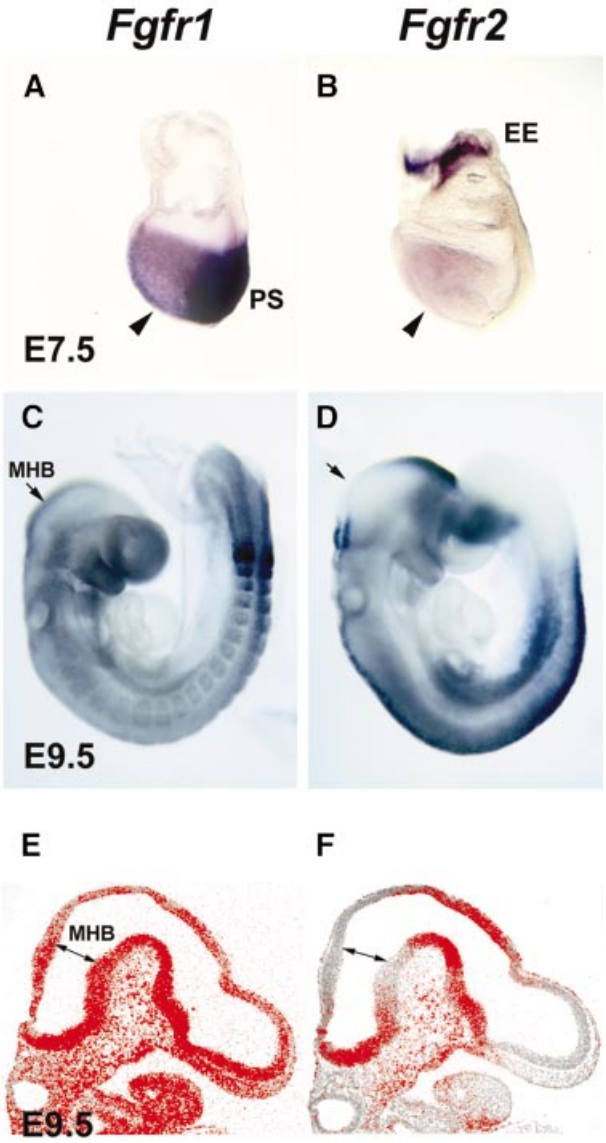

Fig. 1. Expression of *Fgfr1* and *Fgfr2*. Whole-mount *in situ* hybridization analysis of the expression of (**A**) *Fgfr1* and (**B**) *Fgfr2* at E7.5. Expression of both genes is detected in the headfolds (arrowheads). In addition, *Fgfr1* is strongly expressed in the primitive streak region (PS) and *Fgfr2* in the extra-embryonic ectoderm (EE). At E9.5, *Fgfr1* is widely expressed (**C**), whereas *Fgfr2* expression is not detected in the anterior rhombomere 1 and the midbrain (**D**). *In situ* hybridization analysis of (**E**) *Fgfr1* and (**F**) *Fgfr2* expression on sagittal sections of E9.5 embryos. *Fgfr1* is widely expressed in the neural tube, whereas *Fgfr2* appears to be absent from the tissue around the isthmus. Arrows in (C)–(F) mark the midbrain–hindbrain boundary (MHB).

mutants, suggesting that a midbrain-derived signal in turn maintains *Fgf8* (Lee *et al*., 1997). Other FGF-regulatable genes include *Pax2/5* and *En1/2*, which are expressed around the isthmic organizer in a graded manner. These signaling molecules and transcription factors appear to be involved in a complex regulatory network responsible for the maintenance of their expression and development of the midbrain–hindbrain region (reviewed in Wurst and Bally-Cuif, 2001; Liu and Joyner, 2002).

Cells sense the FGFs by tyrosine kinase-type cell surface receptors. Four FGF receptor (FGFR) genes exist in the mammalian genome (*Fgfr1–Fgfr4*). All of the FGFRs can bind several FGF family members and the receptor–ligand interaction is affected by the proteoglycan co-receptors expressed on the target cell. Studies with mice carrying null mutations in each of the *Fgfr* genes have suggested that two of these, *Fgfr1* and *Fgfr2*, carry out the majority of FGF receptor functions during early embryonic development. Embryos homozygous for a *Fgfr1*-null allele fail in gastrulation (Deng *et al*., 1994; Yamaguchi *et al*., 1994), and FGFR1 has been suggested to regulate adhesive and migratory properties of mesodermal cells during their traversal of the primitive streak (Ciruna and Rossant, 2001). In addition to the primitive streak, *Fgfr1* is widely expressed in other embryonic tissues, including developing nervous system (Yamaguchi *et al*., 1992; Walshe and Mason, 2000).

As described above, FGF signaling has been implicated at several stages of development of the mid- and hindbrain. However, the direct target tissues and the receptors of FGF signals are still poorly understood. In the current work we demonstrate by tissue-specific mutagenesis that *Fgfr1* is required after establishment of the midbrain–hindbrain region for the response to the signals from the isthmic organizer. Our results further suggest that FGF signaling through FGFR1 is directly involved in regulation of gene-expression in both the mid- and hindbrain. In addition, FGFR1 appears to be important for specific cell-adhesive characteristics at the midbrain–hindbrain boundary.

Results

Expression of Fgfr1 and Fgfr2 during early development of the mid- and hindbrain

We first analyzed the expression of two potential mediators of isthmic signaling, *Fgfr1* and *Fgfr2*, in mouse embryos by *in situ* mRNA hybridization. At a late headfold stage [embryonic day (E) 7.5], around the stage when the isthmic organizer is induced, *Fgfr1* expression was detected in the head folds (Figure 1A). Prominent expression was also detected in other regions of the developing embryo, including the primitive streak. These results are consistent with the earlier studies of *Fgfr1* expression (Yamaguchi *et al*., 1992). At E7.5, *Fgfr2* was also found to be expressed in the head folds (Figure 1B). Strong *Fgfr2* expression was also detected in the extra-embryonic ectoderm. Later, at E8.5–9.5, widespread *Fgfr1* expression was observed in the developing central nervous system, including the midbrain–hindbrain region (Figures 1C and E, and 2J and K). *Fgfr2* was detected in the diencephalon, hindbrain and spinal cord, especially in the dorsal region (Figure 1D). In contrast to *Fgfr1*, no *Fgfr2* expression was detected at the midbrain–hindbrain boundary in the anterior rhombomere 1 or posterior midbrain (Figure 1F).

Generation of a conditional Fgfr1 allele

To study the function of *Fgfr1* in the development of the mid- and hindbrain, we wanted to inactivate it in a tissue-specific fashion. To generate a *Fgfr1* allele, $Fgfr1^{flox}$, which can be inactivated by the site-specific recombinase Cre, we employed targeting vectors described earlier (Partanen *et al*., 1998). Using these vectors and transient Cre expression in embryonic stem cells, we sequentially introduced recognition sites of Cre (loxP sites) into two

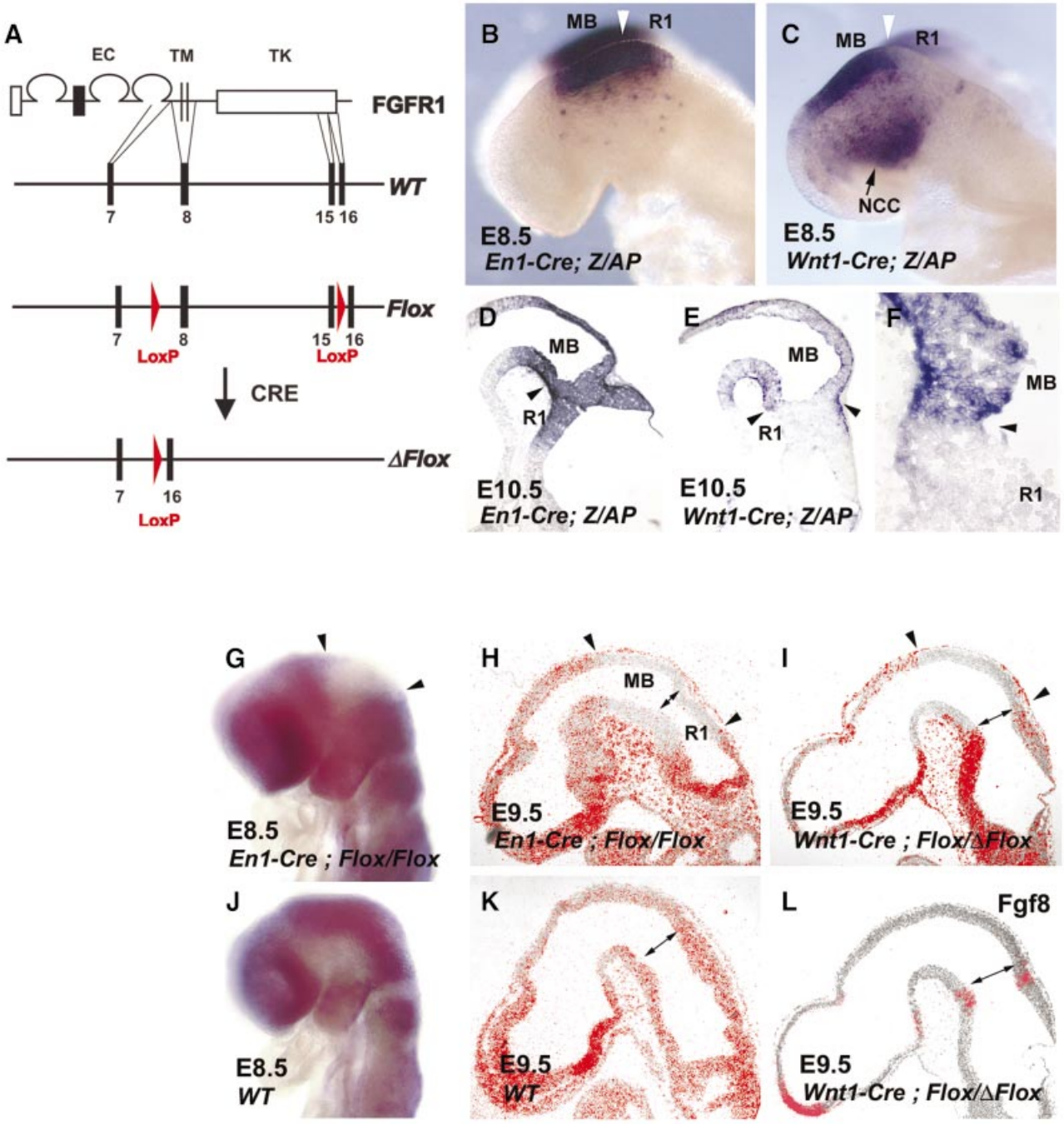

Fig. 2. The conditional *Fgfr1* allele, *Fgfr1*^{flox}, and its inactivation by *En1-Cre* and *Wnt1-Cre*. (**A**) Schematic presentation of the *Fgfr1*^{flox} allele and its inactivation by the Cre-recombinase. The structures of the FGFR1 protein and the wild-type *Fgfr1* locus are shown at the top. Only exons 7, 8, 15 and 16 are depicted. LoxP sites were introduced into introns 7 and 15 by sequential gene targeting to generate the *Fgfr1*^{flox} allele. Cre-mediated recombination of the *Fgfr1*^{flox} deletes the transmembrane and most of the intracellular region encoding exons resulting in the inactive *Fgfr1*$^{\Delta flox}$ allele. EC, extracellular domain; TM, transmembrane domain; TK, tyrosine kinase domain. To characterize the Cre activity expressed by the *En1-Cre* and *Wnt1-Cre* mice, they were crossed with mice carrying a *Z/AP* reporter allele. (**B**) Cre-mediated recombination between LoxP sites in the *Z/AP* allele results in alkaline phosphatase (AP) expression in the midbrain (MB) and rhombomere 1 (R1) of an E8.5 *En1-Cre/+; Z/AP/+* embryo. (**C**) In an E8.5 *Wnt1-Cre/+; Z/AP/+* embryo, AP activity is detected in the midbrain and neural crest cells (NCC). Frozen sections of E10.5 (**D**) *En1-Cre/+; Z/AP/+* and (**E** and **F**) *Wnt1-Cre/+; Z/AP/+* embryos are shown. In the *En1-Cre/+; Z/AP/+* embryos, AP was expressed in the midbrain and rhombomere 1. In *Wnt1-Cre/+; Z/AP/+* embryos, AP activity was detected in the midbrain and scattered cells of the rhombomere 1. A boundary is observed between the AP-positive midbrain and mostly AP-negative rhombomere 1 (F). Arrowheads in (B)–(F) point to the midbrain–hindbrain boundary. (**G**–**L**) Inactivation of *Fgfr1* expression by *En1-Cre* and *Wnt1-Cre*. Whole-mount *in situ* hybridization analysis of E8.5 (10 somite stage) *En1-Cre/+; Fgfr1*$^{flox/flox}$ (G) and wild-type (WT) embryos reveals inactivation of *Fgfr1* transcription in the midbrain–hindbrain region by *En1-Cre* (arrowheads). *In situ* hybridization analysis of *Fgfr1* expression in sagittal sections of E9.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ (H), *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ (I) and wild-type (K) embryos. In *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos, inactivation of *Fgfr1* expression occurs both in the midbrain and rhombomere 1, whereas in *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos rhombomere 1 still expresses *Fgfr1*. Regions of affected *Fgfr1* expression are indicated by arrowheads (H and I). A parallel section to the one shown in (I) hybridized with the *Fgf8* probe (L). A double-headed arrow in (H)–(L) marks the midbrain–hindbrain boundary.

different introns of the *Fgfr1* gene (Figure 2A; and Supplementary figure 1 available at *The EMBO Journal* Online). In the resulting allele, $Fgfr1^{flox}$, exons 8–15 encoding the transmembrane domain, juxtamembrane domain and most of the tyrosine kinase domain of FGFR1, are flanked by two loxP sites. Mice hetero- or homozygous for the $Fgfr1^{flox}$ allele are phenotypically indistinguishable from their wild-type littermates. Thus, the introduced loxP sites themselves do not appear to interfere with *Fgfr1* expression.

To test the functionality of the $Fgfr1^{flox}$ allele, we crossed the $Fgfr1^{flox}$/+ mice with mice carrying a *Pgk-Cre* transgene driving ubiquitous Cre expression (Lallemand *et al*., 1998). In mice heterozygous for both the $Fgfr1^{flox}$ allele and the *Pgk-Cre* transgene, recombination between the loxP sites resulted in excision of the genomic DNA flanked by the loxP sites, generating a novel allele, $Fgfr1^{\Delta flox}$. The mice heterozygous for the $Fgfr1^{\Delta flox}$ allele were normal. Thus, the remaining *Fgfr1* sequences in the $Fgfr1^{\Delta flox}$ do not appear to drive expression of dominant-negative gene products, which could significantly interfere with FGF signaling. Embryos homozygous for the $Fgfr1^{\Delta flox}$ allele have gastrulation defects and die at around E9.5, closely resembling the *Fgfr1*-null mutants reported previously (Deng *et al*., 1994; Yamaguchi *et al*., 1994; data not shown). Therefore, $Fgfr1^{flox}$ behaves as a conditional allele, which can be fully inactivated by the Cre recombinase.

Tissue-specific inactivation of Fgfr1 in the midbrain–hindbrain and midbrain

To study the role of FGFR1 in the isthmic organizer signaling, we wanted to inactivate *Fgfr1* in the neuroepithelium of the mid- and hindbrain after their regional specification. For this purpose, we used the *En1-Cre* mice, which express the Cre recombinase from the *En1* locus (Kimmel *et al*., 2000). We also wanted to inactivate *Fgfr1* specifically in the midbrain. For this, we used mice carrying a transgene expressing Cre recombinase under the *Wnt1* promoter (Danielian *et al*., 1998). To analyze the patterns of Cre activity in the *En1-Cre* and *Wnt1-Cre* mice, we first crossed them with the *Z/AP* reporter mouse line (Lobe *et al*., 1999). The *Z/AP* reporter allele was observed to be recombined efficiently and specifically in the midbrain–hindbrain region of the *En1-Cre/+; Z/AP/+* embryos already at the 8 somite stage (E8.5; Figure 2B). Analyses at E9.5 and E10.5 revealed efficient recombination both in the midbrain and entire rhombomere 1 (Figure 2D; data not shown). Efficient Cre-mediated recombination of the *Z/AP* allele was also detected in the midbrain of *Wnt1-Cre/+; Z/AP/+* mice. In contrast, except for a few scattered cells, cells in the rhombomere 1 carried mostly the unrecombined *Z/AP* allele. At E9.5–12.5, all the neuroepithelial cells of the midbrain appeared to carry the recombinant allele (Figure 2E and F; data not shown). Although isolated recombinant cells were also observed in the rhombomere 1 (Figure 2E), a boundary between recombinant cells in the midbrain and mostly unrecombinant cells in the hindbrain could be observed (Figure 2E and F).

We then crossed the $Fgfr1^{flox}$ mice with the *En1-Cre* and *Wnt1-Cre* mice to inactivate *Fgfr1* in a tissue-specific manner. To determine the pattern of Cre-mediated recombination and inactivation of the $Fgfr1^{flox}$ allele, we carried out *in situ* hybridization analyses of *En1-Cre/+;* $Fgfr1^{flox/flox}$ and *Wnt1-Cre/+;* $Fgfr1^{flox/\Delta flox}$ embryos with a *Fgfr1* cDNA probe containing exonic sequences between the loxP sites in the $Fgfr1^{flox}$ allele. In *En1-Cre/+;* $Fgfr1^{flox/flox}$ embryos at E8.5, reduction in *Fgfr1* signal was observed already at the 8 somite stage, and at the 10–11 somite stage the midbrain–hindbrain region appeared negative for *Fgfr1* expression (Figure 2G and J; Supplementary figure 3). Radioactive *in situ* hybridization analysis on tissue sections of *En1-Cre/+;* $Fgfr1^{flox/flox}$ embryos at E9.5 further demonstrated that midbrain and the entire rhombomere 1 were negative for *Fgfr1* expression (Figure 2H and K). In contrast, the *Wnt1-Cre/+;* $Fgfr1^{flox/\Delta flox}$ embryos lacked *Fgfr1* expression in the midbrain, but still expressed abundant *Fgfr1* in the rhombomere 1 (Figure 2I and L). Thus, our experimental approaches allow us to inactivate *Fgfr1* by E9.5 in the midbrain–hindbrain and midbrain using the *En1-Cre* and *Wnt1-Cre* mice, respectively.

Table I. Behavioral analysis of *Fgfr1* midbrain–hindbrain mutants

	Wild type (n = 12)	*En1-Cre/+; Flox/Flox* (n = 11)	P
Rotarod assay			
t [mean (SD)][a]	176 (59)	49 (79)	<0.001
Stationary beam assay			
t [mean (SD)][a]	60 (1.4)	4.3 (5.4)	<0.001
d [mean (SD)][b]	90 (80)	0.40 (1.0)	<0.005

[a]Time on rotarod/beam (s).
[b]Distance walked on beam (cm).

Inactivation of Fgfr1 in the mid- and hindbrain results in ataxia

The majority of *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice survived until adulthood. However, they were visibly uncoordinated, showing abnormal gait and wide stance. The impaired motor coordination of the *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice (n = 11) was further demonstrated by behavioral tests, including stationary beam and rotarod assays (Table I).

Cerebellar and midbrain defects caused by inactivation of Fgfr1 in the midbrain–hindbrain region

Possibly contributing to the behavioral defects, we observed severe abnormalities in the cerebellum of the *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice. In adults, the vermis of the cerebellum was completely absent (n = 5; Figure 3A–D). The cerebellar hemispheres were present, although their foliation was abnormal (Figure 3E and F). The defect in the cerebellar vermis was also obvious in the newborn *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice (n = 3). In addition to the cerebellum, extensive deletions including the inferior colliculi were evident in the posterior midbrain (Figure 3G, H, J and K). A comparable phenotype was observed in newborn *En1-Cre/+;* $Fgfr1^{flox/\Delta flox}$ mice (n = 2; data not shown).

We also analyzed the development of the midbrain and cerebellum in mice homozygous for the hypomorphic *Fgfr1* alleles, $Fgfr1^{n7}$ and $Fgfr1^{n15YF}$, in which the *Fgfr1*

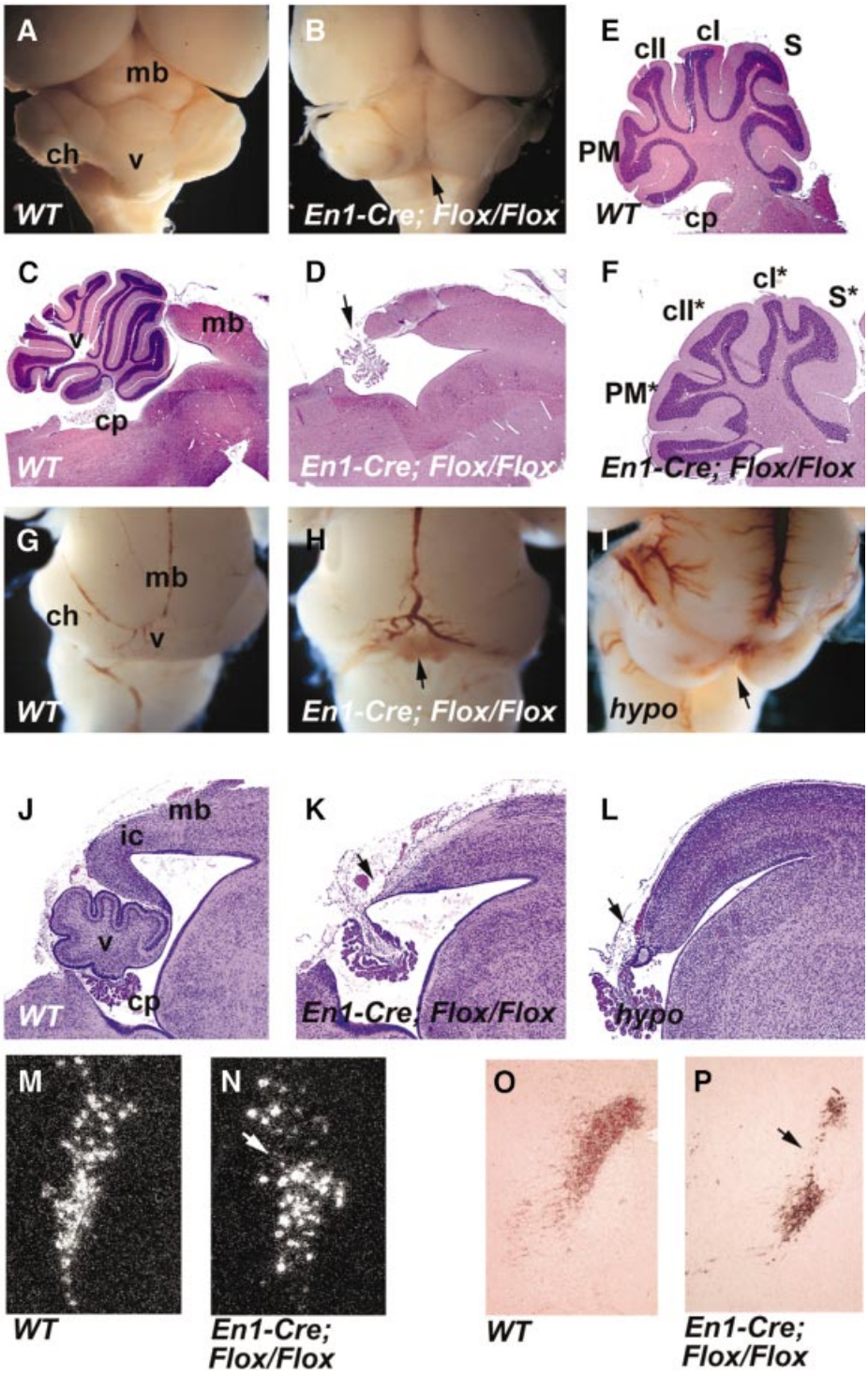

Fig. 3. Morphology of the midbrain and cerebellum of the midbrain–hindbrain-specific and hypomorphic *Fgfr1* mutants. Whole-mount view of (**A**) wild-type and (**B**) *En1-Cre/+; Fgfr1*$^{flox/flox}$ adult brains. The entire vermis is missing in *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice (arrow). (**C** and **D**) Midsagittal and (**E** and **F**) parasagittal sections of adult wild-type (C and E) and *En1-Cre/+; Fgfr1*$^{flox/flox}$ (D and F) brains. Complete aplasia of the vermis is evident in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice [arrow in (D)]. The *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice have cerebellar hemispheres, but their pattern of foliation is altered (F). Whole-mount views and corresponding midsagittal sections of new-born wild-type (**G** and **J**), *En1-Cre/+; Fgfr1*$^{flox/flox}$ (**H** and **K**) and *Fgfr1*$^{n15YF/n15YF}$ (**I** and **L**) mice. Aplasia of the vermis and deletions of the inferior colliculi can be seen in both types of *Fgfr1* mutants (arrows). Locus coeruleus, identified by *Dopamine-β-hydroxylase* mRNA *in situ* hybridization of adult brain sections (**M** and **N**) and anti-tyrosine hydroxylase immunostaining of newborn brain sections (**O** and **P**) appears disorganized in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mutants (N and P) compared with wild type (M and O). cI, crus I; cII, crusII; ch, cerebellar hemisphere; cp, choroids plexus; ic, inferior colliculus; mb, midbrain; PM, paramedian lobule; S, simplex; v, vermis. The mutant hemisphere lobes are labeled with asterisks.

transcript levels are reduced by ~80 and 90%, respectively (Partanen *et al.*, 1998). Defects in the midline vermis and partial deletions of the inferior colliculi of the midbrain were observed in both *Fgfr1*$^{n7/n7}$ (n = 8) and *Fgfr1*$^{n15YF/n15YF}$ (n = 10) newborn mice (Figure 3I and L; data not shown).

In addition to the dorsal midbrain and cerebellum, we analyzed several ganglia in the alar and basal plates of the midbrain–hindbrain region of *En1-Cre/+; Fgfr1*$^{flox/flox}$ mutants, including locus coeruleus, substantia nigra, cranial nerves III and IV, pontine nucleus, the nucleus pedunculoponinus tegmentalis and nucleus parabigeminalis. Tyrosine hydroxylase-positive neurons were found in newborn and adult mutants, in both the substantia nigra of the midbrain and the locus coeruleus of the rhombomere 1. Also, *Dopamine-β-hydroxylase* mRNA *in situ* hybridization revealed locus coeruleus in the mutants. However, locus coeruleus appeared to be disorganized compared with the wild type (Figure 3M–P). Whole-mount neurofilament staining of E10.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos revealed both the oculomotor (III) nerve from the midbrain and trochlear (IV) nerve from the anterior rhombomere 1. Consistently, the oculomotor and trochlear nuclei also appeared unaltered in adult mutants. In addition, pontine nuclei as well as mesopontine nuclei such as nucleus pedunculopontinus tegmentalis and nucleus parabigeminalis were found to be present in the mutants (Supplementary figure 2; data not shown). Thus, in contrast to the dorsal brain structures, no extensive deletions or alterations were observed in the basal plate of the midbrain–hindbrain region.

Inactivation of Fgfr1 in the midbrain–hindbrain region does not affect cellular survival at E9.5

In order to understand the developmental basis for the deletions in the dorsal midbrain and cerebellum, we analyzed the pattern of cell death in *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos. TUNEL analysis of apoptosis at E9.5 revealed no statistically significant difference in the number of apoptotic cells in the midbrain–hindbrain region of *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos (n = 10) compared with the wild type (n = 8). In addition, analysis of semi-thin sections of E9.5 mutants (n = 3) and whole-mount Nile Blue staining of E10.5 mutants (n = 2) showed no difference in the cellular viability compared with wild-type littermates (data not shown). Therefore, although we cannot completely rule out any effect on the apoptotic index in the midbrain–hindbrain region, cell death at around E9.5 does not appear to be a major component of the phenotype of *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos.

Fgfr1 is required for sustained expression of isthmic organizer dependent genes

To analyze how the mid- and hindbrain-specific inactivation of *Fgfr1* affects the early development of the isthmic region, we analyzed gene expression in E8.5–10.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos by whole-mount *in situ* hybridization. At E8.5 (8–10 somite stage), *Pax2*, *Wnt1* and *Sprouty1*, a FGF-inducible inhibitor of receptor tyrosine kinase signaling (Minowada *et al*., 1999), were expressed in *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos at a level and pattern comparable to the wild type (Figure 4A and B; data not shown). At E9.5, an anterior marker *Otx2* (Figure 4C) and a posterior marker *Gbx2* (Figure 4D) were expressed in the correct domains in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos. In addition, *Fgf8* was expressed in the anterior hindbrain of E9.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos (Figure 4E), although its expression decreased at later stages especially in the dorsal isthmus (data not shown). Thus, the expression of a specific set of genes, including signaling molecules thought to be important for isthmic organizer activity, was established around the midbrain–hindbrain boundary in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos at E8.5–9.5.

In contrast to E8.5, expression of several genes thought to depend on isthmic signals failed to be maintained at later stages. Down-regulation of *Sprouty1* was first observed at the 12 somite stage and Sprouty expression was completely abolished from the isthmic domain at E9.5 (Figure 4G; Supplementary figure 3). Interestingly, some *Sprouty1* expression was still detected further away from the isthmus in dorsal and ventral regions. Also, expression of *Pax2* was virtually absent by E9.5 (Figure 4H). Other isthmic target genes were down-regulated slightly later. *En1* and *En2* were still expressed at E9.5 (Figure 4I and J). However, their expression rapidly decreased thereafter, and by E10 *En1* was barely detectable in either mid- or hindbrain (Figure 4K). In addition, expression of *Wnt1* was down-regulated in the posterior midbrain, especially in its dorsal region, after E9.5 (data not shown). Interestingly, before the down-regulation of the *Wnt1* signal, *Wnt1* expression was observed as a broadened stripe in the posterior midbrain (Figure 4F). In contrast to the wild-type embryos, in which the *Wnt1*-positive cells formed a tight band next to the *Fgf8* expressing anterior hindbrain, the *Wnt1*-positive and -negative cells were extensively mixed in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos. Also, *Otx2* and especially *Gbx2* (Figure 4D) showed heterogeneous expression borders.

Tissue-specific inactivation of Fgfr1 in the midbrain causes deletion of posterior midbrain and cerebellar abnormalities

To determine whether *Fgfr1* directly regulates development of the midbrain, we crossed the *Fgfr1*^{flox} mice with mice carrying a *Wnt1-Cre* transgene. The *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice die neonatally, possibly due to defects in the craniofacial neural crest (Trokovic *et al*., 2003). Analysis of the brains of the newborn *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice (n = 5) revealed deletion of the inferior colliculi of the midbrain, reminiscent of the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice (Figure 5A–D). Development of the dorsal cerebellum was also abnormal. However, in contrast to the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice, the vermis was not completely missing although it was severely malformed in the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mutants (Figure 5C–F). Some cellular differentiation was still observed in the mutant vermis by immunohistochemistry with anti-calbindin antibodies (Figure 5F). A comparable phenotype was seen in the *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ mice (n = 3; data not shown).

Fgfr1 is autonomously required in the midbrain for the maintenance of expression of isthmus-regulated genes

We next analyzed isthmic gene expression in *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos at E9.5. As judged by the expression of *Otx2*, *Gbx2* and *Fgf8* (Figure 6A and C; data not shown), the isthmic organizer forms and is correctly positioned in the *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos. However, expression of isthmus-regulated genes was abnormal specifically in the midbrain. In contrast to the

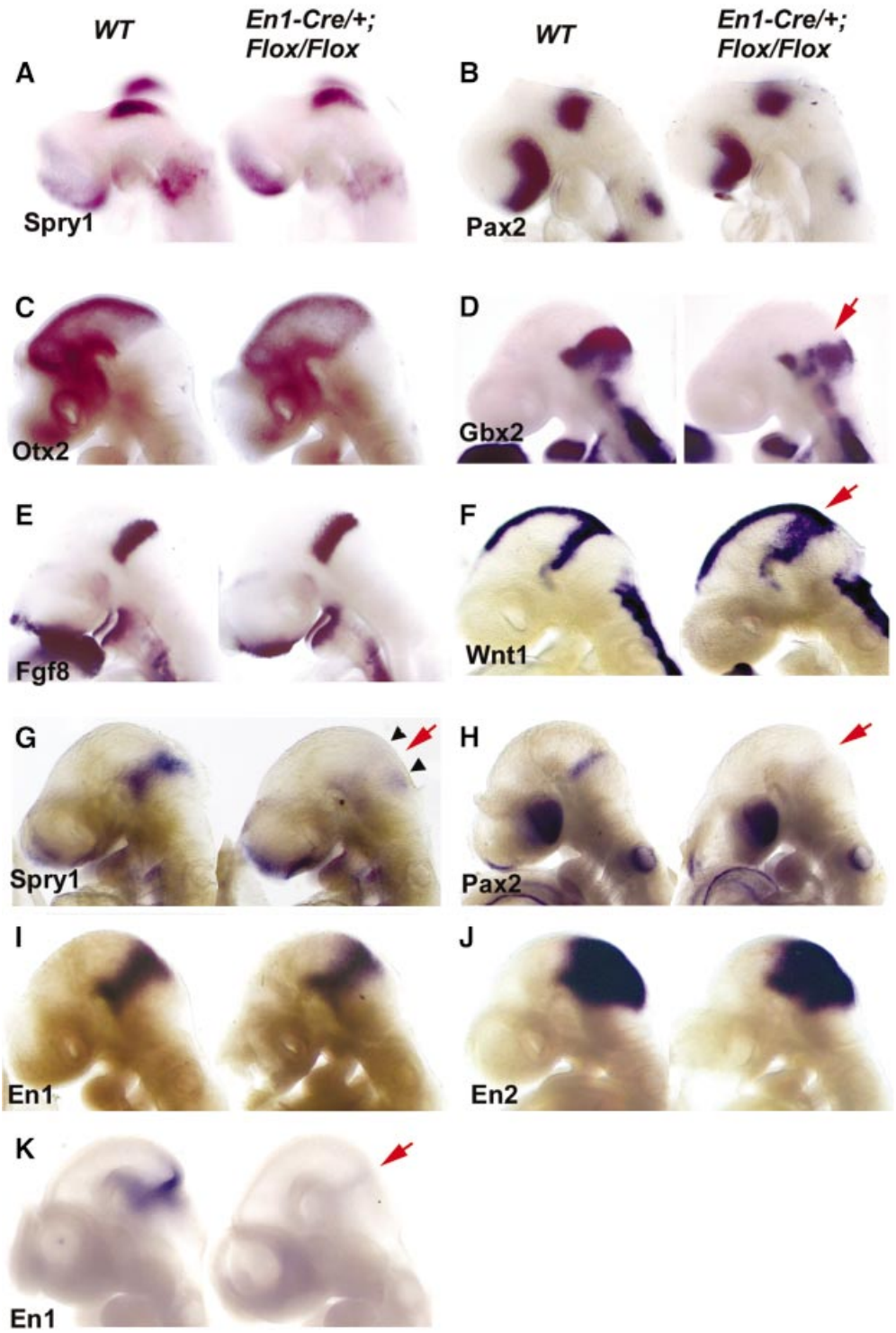

Fig. 4. Analysis of gene expression in the midbrain–hindbrain specific *Fgfr1* mutants. Whole-mount *in situ* hybridization of E8.5 (**A** and **B**), E9.5 (**C**–**J**) and E10 (**K**) wild-type and *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos with *Sprouty1* (A, G), *Pax2* (B, H), *Otx2* (C), *Gbx2* (D), *Fgf8* (E), *Wnt1* (F), *En1* (I, K) and *En2* (J) probes. Red arrows indicate altered gene expression. Small arrowheads in (G) indicate remaining *Sprouty1* expression in regions distal to the isthmus. See text for details.

En1-Cre/+; Fgfr1$^{flox/flox}$ embryos, which showed marked down-regulation of *Sprouty1* in both mid- and hindbrain, E9.5 *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos still expressed *Sprouty1* in the hindbrain, but its expression was down-regulated in the midbrain (Figure 6G). *Wnt1* showed a similar pattern of expression as in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mutants, with early broadened and heterogeneous expression and down-regulation dorsally after E9.5 (Figure 6E and F). The initial broadening of *Wnt1* expression was accompanied by slightly expanded expression of *En1* and *En2* (Figure 6I and J). *En1* was thereafter down-regulated specifically in the midbrain by E10

(Figure 6K). In contrast to the *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos, expression of *Pax2* was still detected as a fuzzy band at the isthmus (Figure 6H). Interestingly, the posterior border of *Otx2* expression, as well as the anterior border of *Gbx2* and *Fgf8* expression, was found to be uneven, showing mixing of positive and negative cell populations in *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos (Figure 6B and D).

Fgfr1 regulates expression of PB-cadherin, an isthmic cell adhesion molecule

To gain insight into the mechanisms responsible for enhanced cell mixing in the *Fgfr1* mutants we next analyzed expression of genes thought to mediate cell adhesion and repulsion. One of these is *Ephrin-A5*, which is specifically expressed in the midbrain. No change in *Ephrin-A5* expression could be observed in E9.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos compared with wild type (Figure 7A and B).

Other candidates for regulators of cellular dispersion include *Cadherins*, a family of mostly homotypic cell-adhesion molecules. One member of the cadherin family, *PB-cadherin*, has been reported to be expressed in the isthmic region in a pattern overlapping with *Wnt1* expression at E10.5 (Kitajima *et al.*, 1999). As the *Wnt1*-positive cells failed to form a tight band of cells in the *En1-Cre/+; Fgfr1*$^{flox/flox}$ and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos, we wanted to analyze whether the expression of *PB-cadherin* was altered in the isthmus. We found *PB-cadherin* expression at the midbrain–hindbrain boundary of wild-type embryos already at E8.5 (data not shown). At E9.5, *PB-cadherin* was expressed as a stripe throughout the isthmic region (Figure 7C). Weaker expression was detected throughout the midbrain. In contrast, both in *En1-Cre/+; Fgfr1*$^{flox/flox}$ (n = 5; Figure 7D) and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ (n = 4; Figure 7E) embryos, *PB-cadherin* expression was markedly down-regulated, except for a patch of ventral expression.

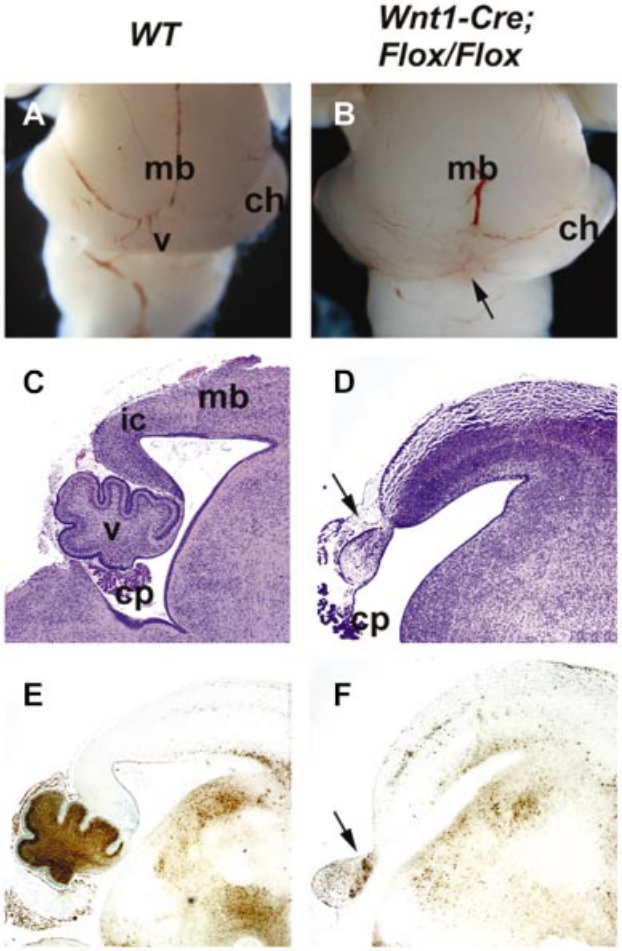

Fig. 5. Morphology of the midbrain and cerebellum of the midbrain-specific *Fgfr1* mutants. Whole-mount views (**A** and **B**) and mid-sagittal sections (**C** and **D**) of brains of newborn wild-type (A and C) and *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ (B and D) mice. Calbindin expression in wild-type (**E**) and *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice (**F**). Arrows indicate the deletion of the inferior colliculi and malformed vermis. ch, cerebellar hemisphere; cp, choroid plexus; mb, midbrain; v, vermis.

Discussion

Genetic loss-of-function studies both in zebrafish (Reifers *et al.*, 1998) and mouse (Meyers *et al.*, 1998; Xu *et al.*, 2000), have demonstrated the importance of FGFs, and FGF8 in particular, in the development of the midbrain–hindbrain region. However, the timing and primary targets of FGF signaling are still unclear. Here we have analyzed the consequences of tissue-specific inactivation of *Fgfr1* in the mid- and hindbrain after the establishment of their regional identity. Loss of *Fgfr1* results in aplasia of cerebellar vermis and inferior colliculi. Our results suggest that FGFR1 is required both in the mid- and hindbrain for their correct response to signals from the isthmic organizer.

Tissue-specific inactivation of Fgfr1

The tissue-specific inactivation of a conditional *Fgfr1* allele, *Fgfr1*^{flox}, was achieved by crosses with *En1-Cre* and *Wnt1-Cre* mice, which allowed apparently complete inactivation of *Fgfr1* transcription by E9.5 in the midbrain and anterior hindbrain. Several additional observations suggest efficient recombination by *En1-Cre* and *Wnt1-Cre*. First, recombination of the *Z/AP* reporter allele occurred by E9.5 in virtually all *En1*- and *Wnt1*-positive cells in the *En1-Cre/+; Z/AP/+* and *Wnt1-Cre/+; Z/AP/+* embryos, respectively (Figure 2; data not shown). Secondly, the phenotypes of *En1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ mice, in which a single recombination event is enough to make a cell null-mutant for *Fgfr1*, are comparable to the *En1-Cre/+; Fgfr1*$^{flox/flox}$ and the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice, respectively. Thirdly, the phenotype of the hypomorphic *Fgfr1* mutants, expressing only ~10% of the wild-type *Fgfr1* mRNA levels, is less severe than the phenotype of the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice. Finally, the early changes in the gene expression in midbrain were similar in *En1-Cre/+; Fgfr1*$^{flox/flox}$ and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos.

Isolated recombinant cells were observed also in the rhombomere 1 in the *Wnt1-Cre/+; Z/AP/+* embryos both at E8.5 and E10.5. It has been proposed that the midbrain–hindbrain border is not a boundary of cell-lineage restriction (Jungbluth *et al.*, 2001). It is therefore possible that the recombinant cells in rhombomere 1 have their origin in the midbrain. Alternatively, the scattered

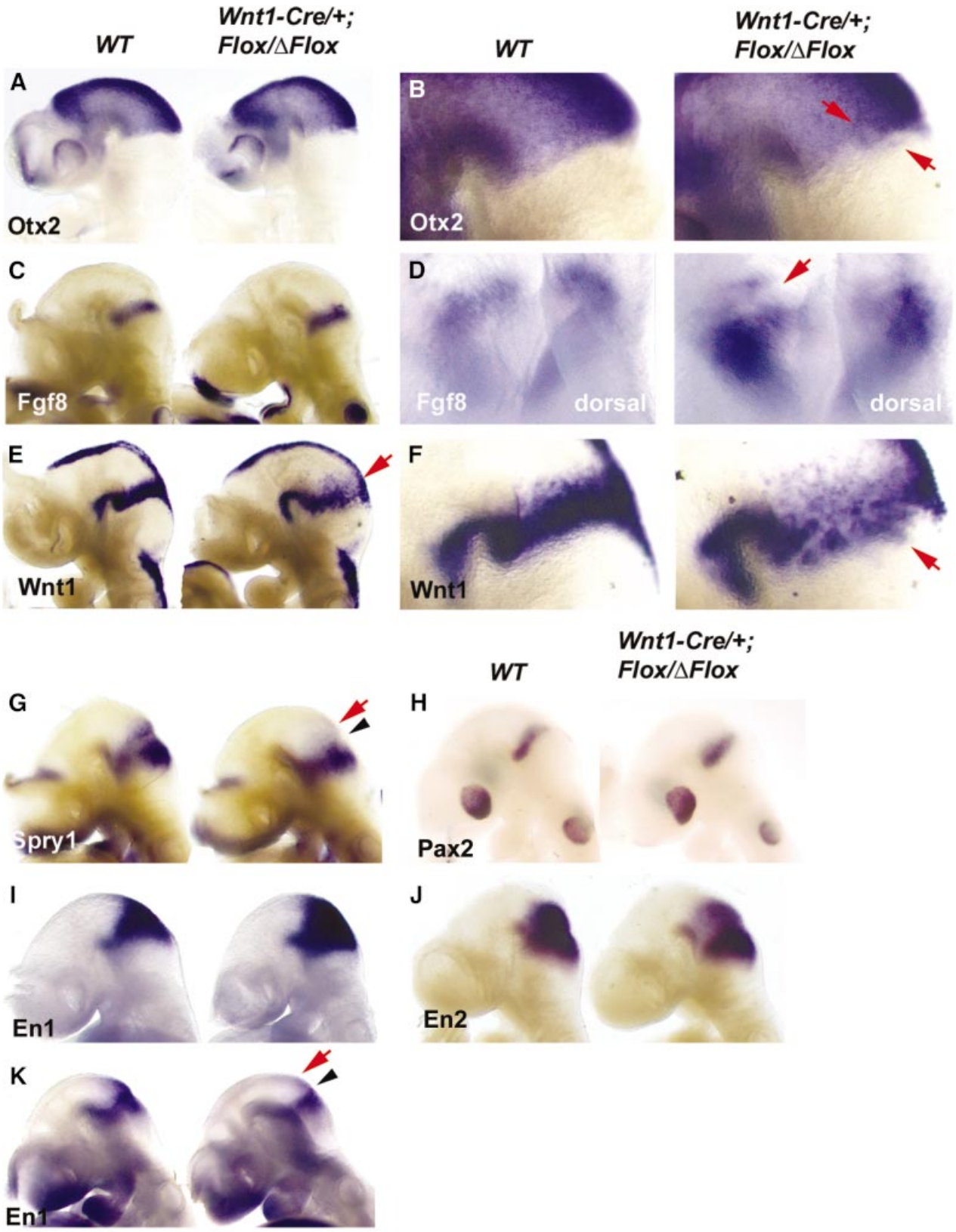

Fig. 6. Analysis of gene expression in the midbrain specific *Fgfr1* mutants. Whole-mount *in situ* hybridization of E9.5 (A–J) and E10.0 (K) wild-type and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos with *Otx2* (**A** and **B**), *Fgf8* (**C** and **D**), *Wnt1* (**E** and **F**), *Sprouty1* (**G**), *Pax2* (**H**), *En1* (**I** and **K**) and *En2* (**J**) probes. Close-up side views of embryos hybridized with *Otx2* (B) and *Wnt1* (F), as well as a slightly oblique dorsal view of embryos hybridized with *Fgf8* probe (D). Arrowheads indicate the isthmus, red arrows indicate altered gene expression. See text for details.

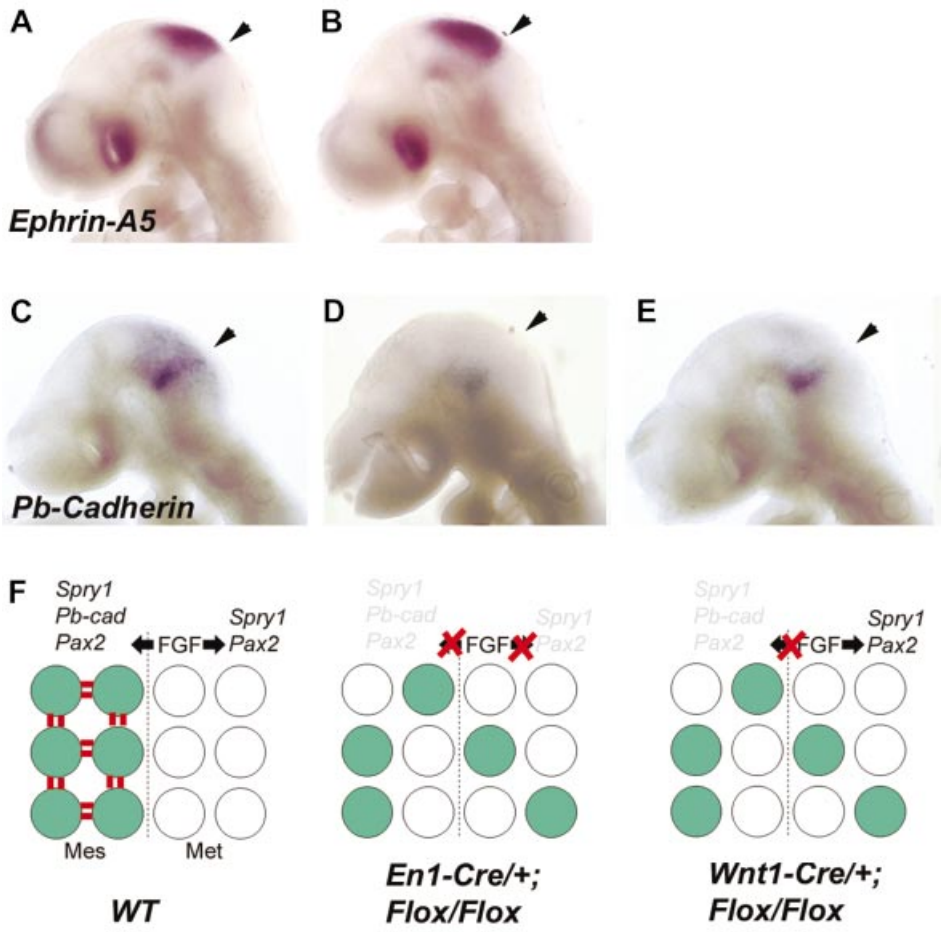

Fig. 7. Expression of *Ephrin-A5* and *PB-cadherin* in *Fgfr1* mutants and a model for FGFR1 function during the maintenance of the isthmic organizer. Whole-mount *in situ* hybridization analysis of *Ephrin-A5* (**A**, **B**) and *PB-cadherin* (**C**–**E**) expression in E9.5 wild-type (A, C), *En1-Cre/+; Fgfr1*$^{flox/flox}$ (B, D) and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ (E) embryos. In both types of *Fgfr1* mutants *PB-cadherin* expression is down-regulated in the midbrain, especially in its dorsal part. Arrowheads in (A)–(E) point to the midbrain–hindbrain boundary. (**F**) A model for the function of FGF signaling through FGFR1 during maintenance of the isthmic organizer. We suggest that FGFR1 regulates gene expression independently in the midbrain (Mes) and hindbrain (Met). Some of the FGFR1-regulated genes confer specific adhesive characteristics to the cells next to the midbrain–hindbrain border.

recombinant cells may result from transient or weak expression of the *Wnt1* promoter in rhombomere 1. The presence of a border between recombinant cells of the midbrain and mostly unrecombined rhombomere 1 suggests that mixing of cells between mid- and hindbrain is already restricted to some extent by E10.5. Our *in situ* hybridization analysis suggested that the majority of the cells in rhombomere 1 still expressed *Fgfr1* in the *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos. We cannot rule out inactivation of *Fgfr1* in isolated cells, which could contribute to observed cerebellar defects in these mutants. However, studies of the expression of both *Fgfr1* and its target genes are consistent with efficient and specific inactivation of *Fgfr1* in midbrain–hindbrain and midbrain by *En1-Cre* and *Wnt1-Cre*, respectively.

Regulation of isthmus dependent gene expression by Fgfr1

In the *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos, *Fgfr1* transcription was inactivated by the 8–10 somite stage. *Sprouty1* is a FGF-responsive gene, which is normally expressed next to the FGF source in several regions of the developing embryo and can be induced by ectopic *Fgf* expression (Minowada *et al*., 1999). Expression of *Sprouty1* in E8.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos suggested that FGF signaling was still active in the isthmic region at this stage. This could be due to expression of residual FGFR1 protein or other *Fgfr*s, perhaps *Fgfr2*, expression of which was detected in the headfolds at E7.5. Initial expression of residual FGFR1 or other FGFRs may also explain why more extensive deletions of midbrain and cerebellum are observed in *Fgf8* mutants (Meyers *et al*., 1998; Reifers *et al*., 1998). It is also possible that *Fgf8* affects multiple target tissues regulating development of the mid- and hindbrain. This is perhaps a less likely explanation, since tissue-specific inactivation of *Fgf8* in the mid- and hindbrain also results in early loss of the entire midbrain and anterior hindbrain (G.Martin, personal communication).

Our results suggest that signaling through *Fgfr1* regulates the maintenance of expression of a set of genes near the isthmic organizer at E9.5. As judged based on isthmus-specific gene expression, the organizer itself is established in both midbrain–hindbrain and midbrain-specific *Fgfr1* mutants, and expression of *Fgf8* and other *Fgfs* (our unpublished observations) is activated in the anterior hindbrain of the mutants. However, FGF signals fail to

elicit normal transcriptional response in the *Fgfr1* mutant target cells. In the E9.5 *En1-Cre/+; Fgfr1*$^{flox/flox}$ mutants, expression of *Sprouty1* and *Pax2* was not detected in the isthmus. This suggests that very little FGF signaling occurs at the midbrain–hindbrain junction of E9.5 embryos in the absence of FGFR1. Thus, at this stage, FGFR1 appears to be the primary FGF receptor receiving isthmic signals and maintaining isthmus-dependent gene expression.

In *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos, expression of genes such as *Sprouty1* and *En1* was specifically altered in the midbrain. This suggests that *Fgfr1* is independently involved in the regulation of gene expression in the midbrain. As these genes are down-regulated in both the mid- and hindbrain of *En1-Cre/+; Fgfr1*$^{flox/flox}$ embryos at the same stage, *Fgfr1* must also have a direct role in the regulation of the anterior hindbrain development.

Later, defects in the vermis of the cerebellum, a derivative of rhombomere 1, were also observed in *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mutants. As discussed above, inactivation of *Fgfr1* in some cells of the hindbrain might contribute to this phenotype. However, the observed gene expression changes in the midbrain, for example down-regulation of *Wnt1*, are likely to also have secondary effects on the cerebellar development in *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice.

Regulation of cell-adhesive properties by Fgfr1?

What could be the molecular and cellular processes regulated by FGFR1? The observed changes in gene expression suggest alterations in cellular identities, i.e. tissue patterning. The behavior of *Wnt1*-positive cells in both midbrain–hindbrain and midbrain-specific *Fgfr1* mutants suggests yet another mechanism. In the wild-type embryos *Wnt1* is expressed as a tight band of cells in the posterior midbrain next to *Fgf8*-expressing cells in the anterior hindbrain. Some mixing of the *Wnt1*-positive and *Wnt1*-negative midbrain cells is observed at the anterior margin of the *Wnt1* expression domain. In the *Fgfr1* mutants, *Wnt1*-positive cells appear to mix extensively with *Wnt1*-negative cells. It is possible that signaling through FGFR1 gives *Wnt1*-positive cells adhesive characteristics, which allow them to sort out of the *Wnt1*-negative cells. In the absence of *Fgfr1*, such characteristics are lost and segregation of *Wnt1*-positive and -negative cells fails. The fact that *Otx2*, *Gbx2* and *Fgf8* also showed heterogenous expression borders suggests that loss of *Fgfr1* also leads to mixing of midbrain and hindbrain cells.

An alternative hypothesis is suggested by the experiments by Jungbluth *et al*. (2001), who demonstrated that cells in the avian midbrain and rhombomere 1 were able to mix with each other. If the midbrain–hindbrain border does not represent a true compartment boundary, FGFR1 may regulate readjustment of identities of the cells traversing the midbrain–hindbrain border. However, previous fate-mapping studies (Millet *et al*., 1996), as well as our analyses of the distribution of genetically marked midbrain cells in *Wnt1-Cre/+; Z/AP/+* embryos, do not support extensive cell mixing at the midbrain–hindbrain boundary. In addition, in chimeric mouse embryos consisting of both wild-type and *Otx2*-null mutant cells, *Otx2*-negative cells were found to segregate from wild-type cells in the midbrain, perhaps due to change in the expression of cell surface adhesion molecules (Rhinn *et al*., 1999). Nevertheless, it is still possible that differential cell adhesion is not a general property of the midbrain and rhombomere 1 cells, but is restricted to the cells at the border of these two domains.

A specific cell adhesion molecule, *PB-cadherin*, is expressed in *Wnt1*-positive cells at the midbrain–hindbrain junction, arguing for unique adhesive properties of these cells (Figure 7; Kitajima *et al*., 1999). Interestingly, our results suggest that *PB-cadherin* is one of the transcriptional targets of FGFR1 signaling, and thus its loss may contribute to the mixing of cells at the midbrain–hindbrain border in the *Fgfr1* mutants (see Figure 7F). In addition to the transcriptional response, it is possible that intercellular signaling pathways of FGFR1 more directly regulate cell-adhesive properties. For example, tyrosine phosphorylation has been reported to regulate the activity of catenins, which in turn affect cadherin function (Lilien *et al*., 2002).

A failure in the segregation between mid- and hindbrain cells has been reported in hypomorphic *Wnt1*^{Sw} mutants (Bally-Cuif *et al*., 1995). Our results suggest that the behavior of the midbrain cells is affected prior to loss of *Wnt1* expression in *En1-Cre/+; Fgfr1*$^{flox/flox}$ and *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos. It is therefore possible that the cell-sorting defect in *Wnt1*$^{Sw/Sw}$ mice results from down-regulation of isthmic FGF signals. The broadening of the *Wnt1* expression domain may also explain the initial slight expansion of the midbrain in *Wnt1-Cre/+; Fgfr1*$^{flox/\Delta flox}$ embryos, as WNT1 has been suggested to stimulate cellular proliferation in the midbrain–hindbrain region (W.Wurst, unpublished data).

An interesting parallel in organizer regulation can perhaps be found in the wing imaginal disc of *Drosophila*. In the wing disc the signaling molecule *Hedgehog*, expressed by the cells in the posterior compartment, not only induces expression of the morphogen *Decapentaplegic* but also affects adhesive properties in the adjacent cells of the anterior compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997). Regulation of adhesive properties by FGF signaling could explain in part how the isthmic organizer is kept as a straight and coherent signaling center, and how the distinct mid- and hindbrain domains are maintained.

Materials and methods

Homologous and site-specific recombination in embryonic stem cells

See Supplementary figure 1 for detailed description of generation of the *Fgfr1*^{flox} allele by gene targeting.

Generation and genotyping of mutant mice

Fgfr1^{flox}/+ embryonic stem cells were aggregated with morula stage embryos of the ICR strain to produce chimeric mice, which transmitted the *Fgfr1*^{flox} allele through the germ line. All the experiments were performed in an outbred (129sv/ICR) background. The *Fgfr1*^{flox} allele was detected by PCR with primers LoxP-1 (5′-AATAGGTCCCTCGACGGTATC-3′) and *Fgfr1* i73 (5′-CTGGGTCAGTGTGGACAGTGT-3′). The wild-type *Fgfr1* allele was detected with the primers *Fgfr1* wt5′ (5′-CCCCATCCCATTTCCTTACCT-3′) and *Fgfr1* wt3′ (5′-TTCTGGTGTGTCTGAAAACAGCT-3′). The different Cre alleles were detected with the primers Cre5′ (5′-AATCTCCCACCGTCAGTACG-3′) and

Cre3′ (5′-CGTTTTCTGAGCATACCTGGA-3′). The Z/AP allele was detected by β-galactosidase staining.

β-galactosidase and alkaline phosphatase staining

β-galactosidase and alkaline phosphatase staining of whole-mount E8.5–10.5 embryos as well as frozen sections was performed as described previously (Lobe *et al*., 1999).

Behavioral analyses

In the rotarod assay, the mice were placed on a rotating rod (diameter 15 cm, speed 15 r.p.m.). The time the mice were able to stay on top of the rod was measured. The experiment was terminated after 5 min. Each mouse was assayed nine times (Table I).

In the stationary beam assay the mice were placed in the middle of a horizontal beam (diameter 2 cm, length 150 cm). The time the mice stayed on the beam and the distance they walked on the beam were measured. The experiment was terminated after 1 min. Each mouse was assayed nine times (Table I).

Histology and immunohistochemistry

Adult tissues were fixed by intracardial perfusion with 4% paraformaldehyde. Brain tissues of newborn mice were dissected in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde. The tissues were dehydrated, embedded in paraffin and sectioned in 5 μm slices. Hematoxylin–eosin was used for counterstaining. Immunohistochemistry on paraffin sections with anti-calbindin (Swant cat. CB38) and anti-tyrosine hydroxylase (Chemicon AB152) antibodies was performed according to standard procedures. For semi-thin sections, embryos were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, overnight at 4°C, post-fixed in 1% osmium tetroxide, and embedded in Epon. One micrometer plastic sagittal sections were cut and stained with toluidine blue.

Analysis of cell death

TUNEL assays. To detect apoptotic cells, TUNEL assays were performed on paraffin sections of E9.5 embryos using the *in situ* cell death detection kit (Roche cat. 1684 795).

Nile Blue sulfate (NBS) staining. Following dissection, embryos (E10.5) were washed in PBS, and incubated for 30 min at 37°C in filtered NBS (Sigma N-5632) saturated water diluted 1:1000 in PBT (PBS containing 0.1% Tween-20). Embryos were then washed several times in PBT at room temperature and photographed immediately.

mRNA in situ hybridization analyses

Whole-mount mRNA *in situ* hybridization of E8.5–10.5 embryos with *En1*, *En2* (Davis and Joyner, 1988), *EphrinA5* (a gift from David Wilkinson), *Fgfr1* (bp 1152–1724 of NM010206), *Fgfr2* (a gift from Alka Mansukhani), *Fgf8* (Crossley and Martin, 1995), *Gbx2*, *Otx2* (Acampora *et al*., 1997), *Pax2* (a gift from Gregory Dressler), *PB-cadherin* (clone ID: UI-M-BH1-akr-h-03-0-UI), *Sprouty1* (a gift from Seppo Vainio) and *Wnt1* (McMahon *et al*., 1992) riboprobes was performed as described previously (Henrique *et al*., 1995). At least four mutants and four littermate controls were hybridized with each of the probes. Hybridization and subsequent treatments of the mutants and the littermate controls were carried out simultaneously in the same vial. Radioactive *in situ* hybridizations on paraffin sections with *Fgf8*, *Fgfr1*, *Fgfr2* and *Dopamine-β-hydroxylase* (bp 620–1018 of S50200) probes were carried out as described by Wilkinson and Green (1990).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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