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# Differentiation of cerebellar cell identities in absence of Fgf signalling in zebrafish Otx morphants

**Isabelle Foucher[1], Marina Mione[2], Antonio Simeone[1,3,4], Dario Acampora[1,3,4], Laure Bally-Cuif[5,6] and Corinne Houart[1,*]**

Although the secreted molecule Fgf8 is a key player of the isthmic organiser function, the mechanisms by which it acts remain unclear. Here, we present evidence indicating that Fgf8 is not instructive in establishing zebrafish cerebellar cell identities, although it is required for proliferation and morphogenesis of this territory. We first show that, as in mouse, lack of Otx function in zebrafish leads to transformation of the presumptive mesencephalon into an extended rhombomere 1 (r1). Expanded *Fgf8* expression was proposed to be the cause of this fate transformation. However, this report demonstrates that zebrafish embryos lacking both Otx and *fgf8* functions retain an extended r1 and display differentiation of at least two cerebellar cell fates. We show that this is not caused by presence of other Fgfs, which implies that in absence of Otx, Fgf function is not necessary for the differentiation of cerebellar cell types. Otx proteins are therefore potent repressors of cerebellar fates, kept out of r1 progeny by Fgf8. Because Otx transcripts are not present in presumptive r1 territory prior to *fgf8* expression, Fgf8 is required to maintain, rather than induce, the posterior boundary of Otx expression. This maintenance is enough to allow cerebellar differentiation.

**KEY WORDS: Cerebellum, Fgf8, Otx2, Isthmic organiser**

## INTRODUCTION

Following neural induction, local organisers act within the neural plate to refine local identities along the anteroposterior axis (Echevarria et al., 2003; Jessell and Sanes, 2000; Lumsden and Krumlauf, 1996). Understanding how these organisers influence their surrounding is a current challenge. As yet, two such local organisers have been described (Houart et al., 1998; Wilson and Houart, 2004; Wurst and Bally-Cuif, 2001). One of these, the isthmic organiser (IsO), placed at the interface between mesencephalon (or midbrain) and metencephalon (comprising rhombomeres 1 and 2; r1, r2), is necessary and sufficient for the development of these structures [also called mes/met domain (Liu and Joyner, 2001; Raible and Brand, 2004; Wurst and Bally-Cuif, 2001)]. The patterning activity of the isthmus was initially demonstrated in avian embryos by transplantation experiments inducing either transformation of part of the caudal forebrain into an ectopic midbrain (Alvarado-Mallart et al., 1990; Martinez et al., 1991), or re-fating of some of the posterior hindbrain into ectopic cerebellum [a major derivative of the metencephalon (Martinez et al., 1995)]. Genetic deletions and mutations of different IsO mouse genes cause the loss of mes/met derivatives (Chi et al., 2003; Lun and Brand, 1998; McMahon and Bradley, 1990; Schwarz et al., 1997; Wurst et al., 1994) and demonstrate that the IsO controls proliferation, maintenance and regionalisation of the mes/met domain. Fgf8, a secreted signalling molecule expressed at the IsO, can mimic the inductive effects of IsO grafts, by regulating cell proliferation and controlling gene expression around the isthmus (Crossley et al., 1996). In addition, like the IsO grafts, local mis-expression of Fgf8 can induce the development of ectopic or enlarged cerebella (Sato et al., 2001; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). However, although transcriptional targets of Fgf signal have been isolated [i.e. feedback-induced antagonists such as Spry4, Pea3, Erm, Sef or Mkp3 (Furthauer et al., 2002; Furthauer et al., 2001; Kawakami et al., 2003; Roehl and Nusslein-Volhard, 2001)], the cellular events directly requiring Fgf8 signalling are yet to be understood. *Fgf8*$^{-/-}$ mouse embryos fail to gastrulate, making it impossible to readily study Fgf8 contribution to the IsO (Meyers et al., 1998; Sun et al., 1999). By contrast, the analysis of a variety of *fgf8* conditional inactivations showed the need for Fgf8 in isthmus formation (Meyers et al., 1998). More recently, Fgf8 lack-of-function studies in zebrafish (Jaszai et al., 2003; Reifers et al., 1998) and mice [conditional Fgf8 mutation inside the mes/met domain (Chi et al., 2003)] revealed that the loss of cerebellum in these mutants is preceded by a fate transformation of presumptive r1 cells into *otx2*-expressing mesencephalic progenitors. In the zebrafish, the lack of gastrulation defect in *fgf8*-null embryos is explained by the presence of maternal Fgf8 protein, allowing the embryo to gastrulate. The zygotic protein present in *ace/fgf8*$^{-/-}$ is fully inactive, and no Fgf activity is detectable in the MHB of *ace/fgf8*$^{-/-}$ mutants (Furthauer et al., 2001) (this study).

Thus, a growing body of evidence present the restriction of the *otx2* expression domain as one of the early functions of Fgf8 inside the IsO (Martinez et al., 1999; Sato et al., 2001; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). Molecular studies, using Fgf8 bead implantation rostral to the isthmus, suggest that the formation of an ectopic midbrain is always associated with a very small cerebellum around the Fgf8 source. These tiny cerebella are thought to be formed by local repression of *otx2* transcripts, creating an artificial boundary of *otx2* expression that would allow formation of an IsO able to repolarise the area (Martinez et al., 1999). The local repression of Otx2 is suggested to be necessary for the induction of

[1]MRC Centre for Developmental Neurobiology, New Hunt's House, King's College London, London SE1 9RT, UK. [2]The FIRC Institute of Molecular Oncology, Via Adamello, 16, 20139 Milano, Italy. [3]CEINGE Biotecnologie Avanzate, Via Comunale Margherita 482, 80145 Naples, Italy. [4]Institute of Genetics and Biophysics–ABT, Via G. Marconi 12, 80125 Naples, Italy. [5]Zebrafish Neurogenetics, GSF-Research Center for Environment and Health, Institute of Developmental Genetics, Ingolstaedter Landstrasse 1, D.85764 Neuherberg, Germany. [6]Institute of Virology, Technical University-Munich, Trogerstrasse 4b, D-81675 Munich, Germany.

*Author for correspondence (e-mail: corinne.houart@kcl.ac.uk)

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*fgf8* in the host cells surrounding the beads. These *fgf8*-positive host cells are, in turn, required for the formation of an ectopic cerebellum (Martinez et al., 1999).

We addressed the relationship between Fgf signal and Otx function in the zebrafish. Reduced level of Otx proteins in *Otx2*$^{+/-}$; *Otx1*$^{-/-}$ or *Otx2*$^{+/-}$; *Otx1*$^{+/-}$ mutant mouse embryos previously unravelled Otx function in positioning the IsO (Acampora et al., 1997; Suda et al., 1997). We obtained hypomorphic Otx lack-of-function (OtxH) embryos using morpholinos against two of the three zebrafish Otx genes. These embryos retain enough Otx function to gastrulate normally but they subsequently show a transformation of the presumptive midbrain territory into an extended r1, resulting in an anterior shift of the isthmus and formation of a bigger cerebellum. In *Otx2*$^{+/-}$; *Otx1*$^{-/-}$ mutant mice, the same transformation was attributed to a rostrally expanded *fgf8* expression domain (Acampora et al., 1997; Suda et al., 1997). Although such an expansion is also observed in OtxH embryos, we show that zebrafish embryos lacking both Otx and Fgf8 functions maintain a transformation of the midbrain tissue into r1 and retain the ability to differentiate cerebellar cell types. This indicates that both early r1 territory and cerebellar cell types are developing in absence of Fgf8, as long as Otx function is also abrogated. This is not due to persistence or upregulation of other Fgf proteins, as we show that OtxH embryos lacking *fgf8* function have no Fgf activity in the presumptive mes/met and that OtxH embryos still develop r1 territory and cerebellar granule cell precursors following complete inhibition of all Fgf signals. Together, our work shows that Fgf8 is not instructive neither in the formation of the r1 territory nor for the differentiation of cerebellar cell identities. Fgf signalling activity is, however, crucial for proliferation, fusion and folding of the cerebellar anlage.

## MATERIALS AND METHODS

### Embryos, in situ hybridisation and immunochemistry

Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). Data presented were obtained from analysis of Kings College London wild-type and of homozygous *acerebellar/fgf8*$^{-/-}$ embryos (Reifers et al., 1998) maintained at 28°C on a 14 hour light/10 hour dark cycle.

Whole-mount in situ hybridisation and immunochemistry were performed using standard procedures (Tallafuss and Bally-Cuif, 2003; Westerfield, 2000), details available upon request. The Engrailed antibody 4D9 was kindly provided by Simon Hughes.

### Fgf signal inhibition

SU5402 (20 μM, Calbiochem) was added on embryos kept at 31°C from 50% epiboly stage until fixation. Efficiency of the treatment on wild-type embryos is measured by the absence of isthmic constriction similar to the one observed in *ace* embryos. A typical reduction in the length of the yolk extension and the tail is also seen after SU5402 treatment (Fig. 7E,F) but not in *fgf8*$^{-/-}$ embryos, as several other Fgfs are expressed and have redundant functions in tail bud. Loss of *spry4* expression after 1 hour of incubation at 31°C was used to confirm treatment efficiency (not shown).

### Morpholinos and injection

MOs (Gene Tools) were designed against *otx1* (GenBank Accession Number BC045290), *otx2* (NM_131251) and *otx1-like* (D26174), diluted in Danieau's media and injected at the one- to four-cell stages. Embryos received 1.2 ng of each MO (0.133 mM, 0.133 pmol/embryo). Morpholinos were as follows: Otx1 MO1-Fluo (complementing bases 417-441), 5′-TGAGGTATGACATCATGCTAGAGGC-3′; Otx2 MO-Fluo (complementing bases 261-285), 5′-GTTGCTTGAGATACGACATCATGCT-3′; Otx1-like MO-Fluo (complementing bases 225-249), 5′-GAGGTATGACATCATTGTTGAGCCC-3′; Otx1 MO2-Bare (complementing bases 375-379), 5′-GATCTTGCCAATAGTTCGATTTCCC-3′; Otx1MO3-Bare (complementing bases 350-374), 5′-CCGATGTTGCAGTTTGACGAAGGAC-3′.

Most of our morpholinos are labelled with fluorescein to facilitate the selection of equally injected embryos. Under these conditions, we observed the phenotype described here in around 98% of the injected embryos, while we observed a range of milder phenotypes in ~5% of all injected embryos in the absence of selection. Each experiment was repeated two to six times, with highly similar results.

To control for the specificity and efficiency of our MOs, we cloned all three full-length Otx cDNAs, including 150 bp of their 5′ sequence. As previously reported in *Xenopus*, overexpression of Otx after RNA injection leads to cell movement defects during gastrulation (*otx1* RNA 80% affected, *n*=52; *otx2* RNA 88% affected, *n*=44; *otx1-like* RNA, 88.2% affected, *n*=34) (see also Bellipanni et al., 2000). The vast majority of embryos injected with a given Otx RNA and its corresponding MOs showed a rescue [*otx1*RNA+*Otx1-1* MO, 80.6% of wild type (*n*=31); *otx2* RNA+*otx2-1* MO, 63.8% of wild type (*n*=40); *otx1-like* RNA+ *otx1like-1* MO, 60.4% of wild type (*n*=43).

As previously reported, *fgf8*$^{-/-}$ mutant larvae are of two different phenotypic classes. Type A larvae (65%, *n*=78) show a tectum slightly altered in its shape but not affected in its length, whereas type B (35%) larvae are overall retarded most probably as a secondary effect of variably abnormal blood circulation. Some of those embryos (generally 10%) with oedema were removed from our analysis of 5-day-old embryos.

### Western blot

Total extracts were prepared from heads of E10.5 mouse or prim-5 zebrafish (10 embryos), processed for standard western blot assay and probed with Otx antibody (Mallamaci et al., 1996) (1/10000). For detection at bud stage, nuclear extracts were prepared from 50 wild-type or OtxH embryos. Coomassie staining was carried out after transfer to compare proteins levels.

## RESULTS

### Generation of OtxH embryos

Otx genes, cognates of the *Drosophila* gap gene *Orthodenticle*, are highly conserved in all vertebrates. Two closely related family members, *Otx1* and *Otx2*, have been shown to be crucial for early brain development in mammals (Acampora et al., 2003; Acampora et al., 2001). To date, three homologues have been described in zebrafish (Mercier et al., 1995). The third zebrafish gene, previously called *otx3* is renamed *otx1-like* as it has a high percentage of similarity with *otx1*. *otx1* and *otx2* are both located on chromosome 17 and *otx1-like* is on chromosome 1 (Ensembl database). The most likely scenario leading to this zebrafish genomic organisation is that the single ancestral Otx gene found in cephalochordates was first tandemly duplicated (Williams and Holland, 1998). Then the two genes were separated in higher vertebrates while an additional whole genome duplication in teleosts led to four Otx gene copies, with a subsequent loss of an *otx2-like* gene.

Gene duplication may have been followed by functional diversification of the different paralogues in zebrafish. To define the function of the three paralogues, we used a battery of morpholino antisense oligomers (MOs) (Nasevicius and Ekker, 2000) targeting zebrafish Otx genes. Only the double knock-down of both *otx1-like* and *otx2* led to a severe defect in the embryonic brain. Western blot analyses, using a polyclonal Otx2 antibody recognising vertebrate Otx1 and Otx2, showed that residual Otx proteins were detectable in these embryos at bud stage, but that the proteins were lost by the end of somitogenesis (Fig. 1A). We called these double knock-down embryos OtxH morphant embryos (H standing for 'hypomorph'). Brain morphology, first analysed in prim-20 live embryos, showed that the telencephalon, olfactory placodes and hypothalamus are present but the epiphysis is missing in OtxH embryos. The patterning defect is also evident caudally where the isthmic constriction is absent. Only one uniform dorsal structure can be observed (arrow in Fig. 1C) instead of a tectum and cerebellum present in wild type embryos (Fig. 1B). As the single Otx2 and

Otx1-like morphants have no visible defect, our data indicate that zebrafish *otx1-like* and *otx2* genes carry a redundant function during early development.

## Transformation of the midbrain into rhombomere 1 in OtxH morphants

Severe reduction of Otx proteins triggers transformation of the mesencephalic anlage into metencephalic derivatives in mouse. We confirmed, by analyzing *mbx* and *mab21L2,* two markers expressed in midbrain and pretectal areas (Kawahara et al., 2002; Kudoh and Dawid, 2001), that this area is dramatically reduced as early as the six-somite stage (Fig. 1F,G; data not shown) and absent by the end of somitogenesis (Fig. 1H,I; data not shown) in OtxH embryos. In contrast to this result, genes expressed across the entire mes/met domain, such as *engrailed* (*En*), *her5* and *pax2.1*, maintain their expression in OtxH embryos until mid-somitogenesis (data not shown). Among those, *En* is the only one steadily expressed in the whole mes/met domain during development and is still partially maintained in prim-5 OtxH embryos (Fig. 1J,K).

To study AP patterning caudal to the midbrain, we analysed *efnb2a* (*ephrinb2a*) expression, which marks presumptive rhombomere1, 4 and 7 (Cooke et al., 2001). From early somite stage, *efnb2a* r1 domain is reproducibly expanded in OtxH embryos (Fig. 2A,B). In order to assess whether this *efnb2a* domain is indeed derived from mes/met descendants, we took advantage of the *her5pac:egfp* line expressing the green fluorescent protein (GFP) under the control of the *her5* regulatory regions (Tallafuss and Bally-Cuif, 2003). *her5* is specifically induced in the entire mes/met

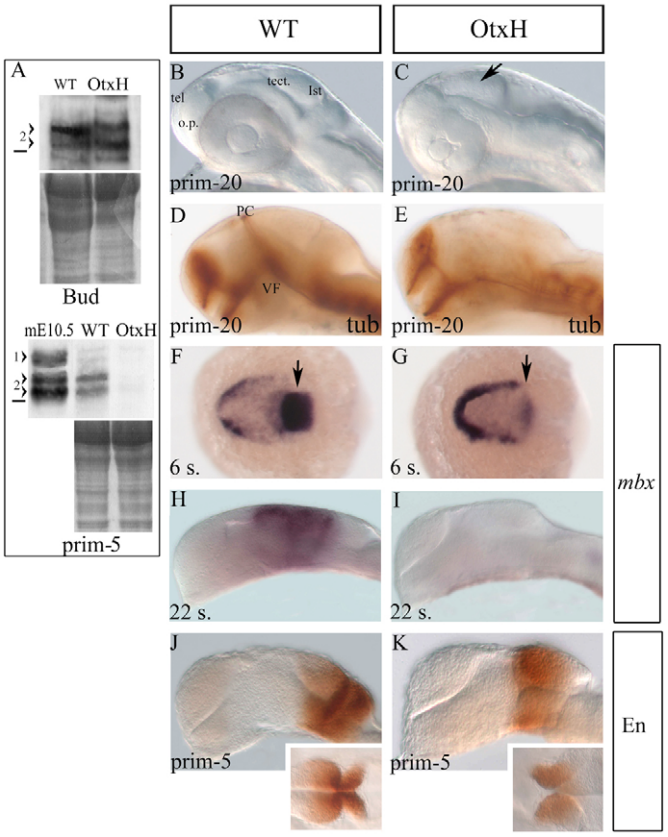


**Fig. 1. Absence of mesencephalon in OtxH embryos.** (**A**) Western blot analysis on wild-type or OtxH zebrafish embryo extracts from end of gastrulation stage (upper panel: bud stage) or late somitogenesis stage (lower panel: prim-5). Proteins staining is shown underneath each to control protein levels between wild-type and OtxH embryos. 1>, mouse Otx1protein; 2>, doublet of mouse or zebrafish Otx2 proteins. The horizontal black line represents the 36.4 kDa marker. In OtxH embryos, Otx2 expression is not visibly affected at the end of epiboly but is lost by the end of somitogenesis. (**B-E**) Lateral views, anterior towards the left, of prim-20 (33 hpf) live brains (B,C) or fixed after acetylated tubulin staining (D,E) from wild-type (B,D) or OtxH (C,E) embryos. The isthmic constriction (arrow in C), the ventral flexure (VF) and the posterior commissure (PC, normally forming at the boundary between forebrain and midbrain) are all absent. tel, telencephalon; o.p., olfactory placode; tect., tectum; ist, isthmus. Dorsal (**F**,**G**) and lateral (**H-K**) views, anterior towards the left, of wild-type (F,H,J) or OtxH (G,I,K) brains. Expression of *mbx* is almost undetectable in the CNS at the six-somite stage in OtxH embryos (arrow in G) compared with wild type (arrow in F) and is lost by the 22-somite stage (I). Expression of Engrailed is detectable in both wild type (J) and OtxH (K) at prim-5 stage. Insets are dorsal views of the isthmic area of the brains shown in J and K.

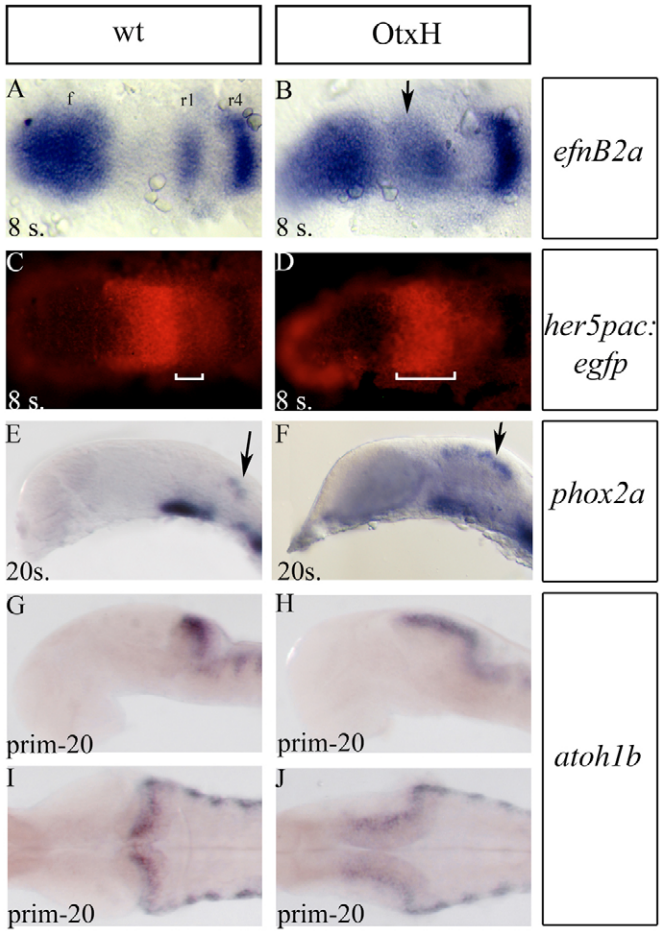


**Fig. 2. Mesencephalon to rhombomere1 transformation in OtxH embryos.** (**A-D**) Dorsal views (anterior towards the left) of eight-somite embryos after double detection of *efnb2a* (blue in A,B) expressed in forebrain (f), rhombomeres 1 (r1), 4 (r4) and 7 (r7) and GFP (red in C,D) in wild type; *her5pac:egfp* (A,C) and OtxH; *her5pac:egfp* (B,D). In OtxH, the mesencephalon is transformed into an expanded r1 territory (white brackets in C and D indicate the anteroposterior extent of *efnb2a*). (**E-J**) Lateral (E-H) and dorsal (I,J) views, anterior towards the left, of wild-type (E,G,I) and OtxH (F,H,J) brains. Locus coeruleus cells expressing *phox2a* (arrows in E,F) and rhombic lip cells expressing *atoh1b* (G-J), which is known to arise from the r1 territory, are expanded in OtxH embryos (F,H,J). I and J are dorsal views of embryos in G and H.

domain during late gastrulation (and completely absent in the rest of the hindbrain). As GFP is a very stable protein, it is able to follow temporally the progeny of the mes/met domain. In *her5pac:egfp* embryos, the metencephalic precursors co-express GFP and *efnb2a*, while the mesencephalic cells express only GFP (Fig. 2A,C). In OtxH/ *her5pac:egfp* embryos, GFP is always co-expressed with *efnb2a* (Fig. 2B,D), showing that the OtxH entire presumptive mes/met domain is transformed into an extended metencephalon.

A number of distinct neuronal populations are generated in r1, including the locus coeruleus (LC) (Crossley et al., 1996; Jaszai et al., 2003) and the cerebellar granule cells (Wingate and Hatten, 1999). The LC is a small group of noradrenergic (NA) neurons, known to be induced by coordinated isthmic Fgf and dorsal midline BMP signals (Guo et al., 1999). In agreement with their commitment as NA lineage, LC neurons express two homeobox genes, *phox2a* and *phox2b*. In 20-somite stage OtxH embryos, this *phox2a*-expressing population, located in the dorsal metencephalon, has at least doubled (arrows in Fig. 2E,F). The origin of cerebellar granule neurons has been microsurgically fate-mapped to r1 in chick (Wingate and Hatten, 1999), and specifically to the embryonic rhombic lip, a specialised proliferative epithelium arising at the interface between the neural plate and the roof plate of the IVth ventricle. To further characterise the nature of the expanded r1 area in morphant embryos, we analysed the expression of markers of cerebellar differentiation. We find that the expanded r1 expresses *atoh1a* and *atoh1b,* two granule cell markers (Adolf et al., 2004; Koster and Fraser, 2001) (Fig. 2G-J; Fig. 4A,B). Finally, zebrin, marker for cerebellar Purkinje cells also shows an expanded population of these cells in OtxH embryos (see Fig. 5I,J). The OtxH transformed mes/met region is therefore differentiating into a large LC and cerebellum. However, the morphology of the enlarged cerebellum is abnormal, mainly owing to an absence of fusion (Fig. 5F,N; Fig. 6F). Some level of disorganisation is also detected in the Purkinje cell layers (Fig. 5J; see also Fig. S1I in the supplementary material).

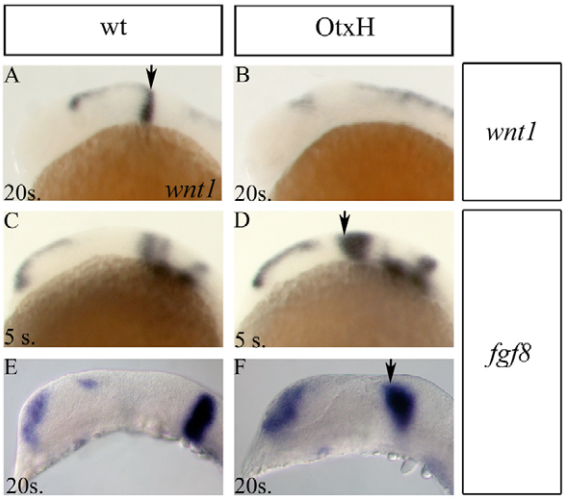


**Fig. 3. Isthmic organiser defects.** Lateral views of wild-type (A,C,E) and OtxH (B,D,F) brains. (**A**,**B**) Most of *wnt1* expression is lost in OtxH embryos; weak remaining expression is often observed anterior to the expanded r1. (**C-F**) *fgf8* expression is expanded in the dorsal mes/met territory of OtxH embryos (arrows) at the 5- (D) and 20-somite stages (F). Arrows indicate the isthmic expression, expanded in the morphants.

## Perturbation of IsO signals upon reduced Otx function

Fate transformation of the mes/met area is likely to be triggered by perturbation of the signalling molecules normally expressed in the IsO. Therefore, we analysed the two MHB secreted factors – *wnt1* expressed just anterior to the Otx/Gbx boundary and *fgf8* just posterior to it (Wurst and Bally-Cuif, 2001) – thereby defining, from early somitogenesis onwards, the midbrain and hindbrain part of the isthmus. In OtxH embryos, an initial residual ventromedial *wnt1* expression is rapidly lost (by the six-somite stage, not shown). Expression then reappears at the 20-somite stage in some dorsal cells located anterior to the expanded r1 (likely diencephalic) and within the roof plate of the posterior neural tube (Fig. 3A,B). Conversely, *fgf8* is improperly activated in the morphants, from the one-somite stage onwards, in a broad area inside the anterior part of the presumptive mes/met domain, while expression in ventral r2 and r4, and within dorsal forebrain appears unaltered (Fig. 3C-F).

We therefore conclude that, in fish, as in mouse (Acampora et al., 1997; Suda et al., 1997), a decreased level of Otx activity leads to the loss of *wnt1* and the expansion of *fgf8* signal inside the mes/met area. In mouse, this *fgf8* upregulation was proposed as the major cause for the local transformation of the midbrain into r1.

## Cerebellar cell fates develop in *fgf8*$^{-/-}$ mutant embryos when Otx function is lowered

Fgf signal is thought to be necessary and sufficient to instruct r1-derived fates. OtxH embryos provide the unique opportunity to test this hypothesis. Unlike OtxH embryos, the *fgf8*$^{-/-}$ mutant [*acerebellar, ace* (Reifers et al., 1998)] lacks cerebellum because of a progressive transformation of the anterior r1 into mesencephalon (Jaszai et al., 2003). Thus, embryos with lowered level of Otx proteins in an *fgf8*$^{-/-}$*/ace* background are expected to lack both msencephalon and r1. Zebrafish *fgf8*$^{-/-}$embryos were injected with the combination of *otx1-like* and *otx2* morpholinos (referred to as *fgf8*$^{-/-}$; OtxH embryos). As previously reported, *atoh1a* is consistently missing from the upper rhombic lip in *fgf8*$^{-/-}$ mutant embryos (Jaszai et al., 2003) and *atoh1a*-expressing cells from the lower rhombic lip are not able to contribute to the cerebellar system (Koster and Fraser, 2001). To our surprise, in *fgf8*$^{-/-}$; OtxH embryos, an upper rhombic lip cell population is restored, resembling the one seen in OtxH embryos (Fig. 4B,D), albeit reduced in size.

To test whether cells from the presumptive posterior rhombic lip may possibly migrate anteriorly to regenerate a cerebellar granule cell population in *fgf8*$^{-/-}$*;* OtxH embryos, we used the stability of GFP protein to follow the origin of the upper rhombic lip cells in *fgf8*$^{-/-}$*; her5pac:egfp*; OtxH embryos. We consistently observe co-localisation of *atoh1a* and GFP on parasagittal cryostat sections of *fgf8*$^{-/-}$; *her5pac:egfp;* OtxH prim5 brains, ruling out the possibility of cell migration from posterior GFP-negative rhombomeres (Fig. 4N,O). In *her5pac:egfp* wild-type embryos, a subpopulation of GFP-positive cells is co-expressing atoh1a, confirming that, as in higher vertebrates, zebrafish granule cells are born within an r1 territory characterised by *her5* expression. This *atoh1a* and GFP co-expressing population is absent in *fgf8*$^{-/-}$ embryos (Fig. 4K,L) and is present in *fgf8*$^{-/-}$*;* OtxH (Fig. 4N,O), as well as in OtxH embryos (Fig. 4H,I).

Differentiation of cerebellar cells is further assessed using *pax6* and reelin staining granule cells (Fig. 5A-H) (Costagli et al., 2002) and Zebrin/AldolaseC marking Purkinje cells (Fig. 5I-L; see Fig. S1 in the supplementary material) (Lannoo et al., 1991; Miyamura and

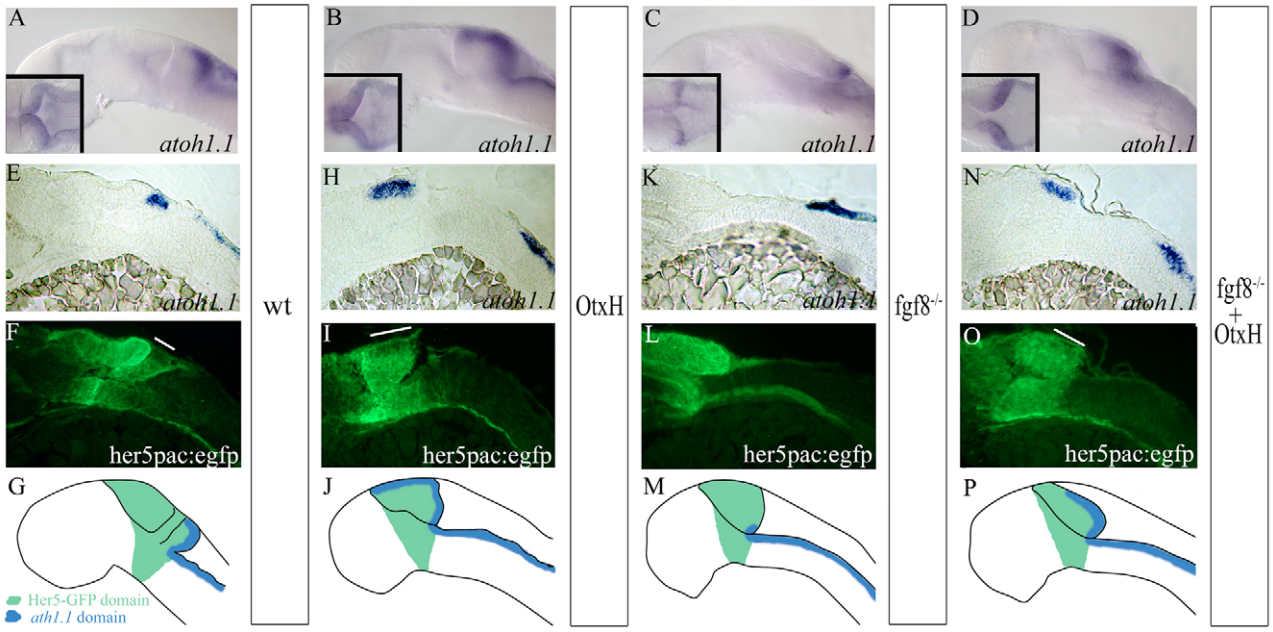


**Fig. 4. Formation of the granule cell population in *fgf8*$^{-/-}$; OtxH embryos.** *atoh1a* expression in wild-type (**A**), OtxH (**B**), *fgf8*$^{-/-}$ (**C**) and *fgf8*$^{-/-}$; OtxH (**D**) whole-mount embryos. MyoD expression in the somite was concomitantly detected to confirm *fgf8*$^{-/-}$genotype. (**E-O**) Para-saggital 10 μm sections, anterior towards the left, of wild-type *her5pac:egfp* (E,F), OtxH (H,I), *fgf8*$^{-/-}$ (K,L) and *fgf8*$^{-/-}$; OtxH (N,O) brains, showing co-localisation of *atoh1a* (E,H,K,N) and GFP expression (F,I,L,O). Granule cells absent in *fgf8*$^{-/-}$ are rescued in *fgf8*$^{-/-}$; OtxH and are GFP positive (white lines on fluorescent pictures represent the length of the *atoh1a* domain in the upper rhombic lip obtained from bright field pictures). (**G**,**J**,**M**,**P**) Cartoons summarising GFP (in green) and *atoh1a* (in blue) expressions in wild-type (G), OtxH (J), *fgf8*$^{-/-}$ (M) and *fgf8*$^{-/-}$; OtxH (P) embryos carrying the transgene *her5pac:egfp*.

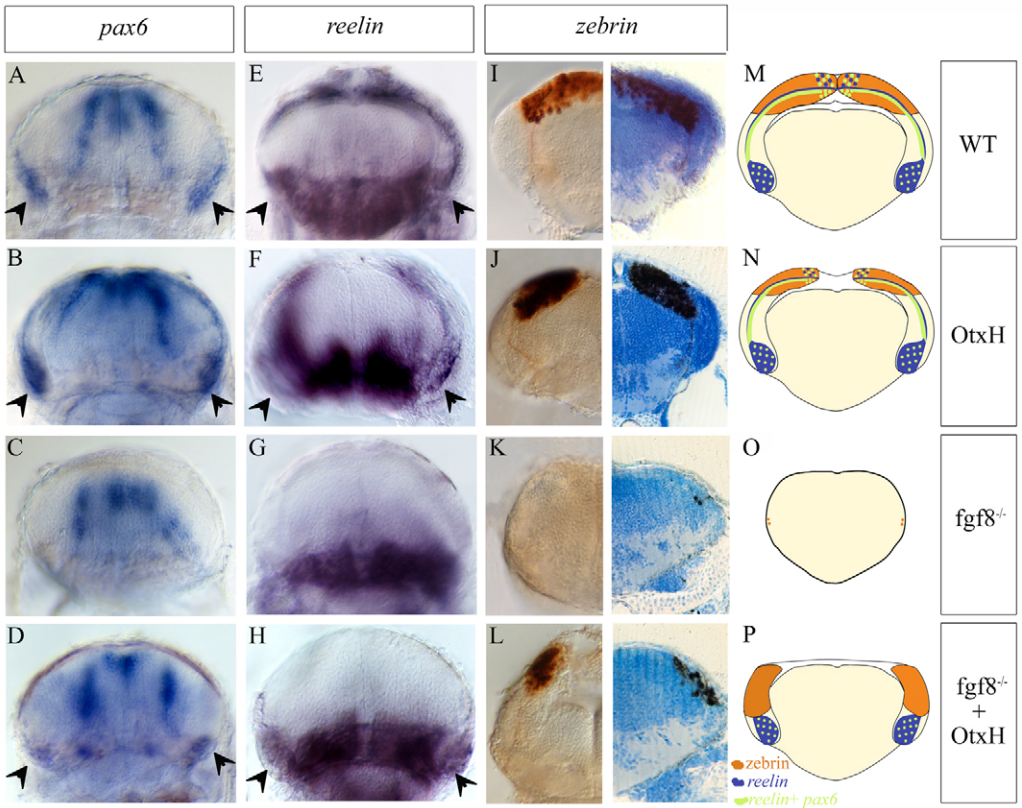


**Fig. 5. Cerebellum formation in *fgf8*$^{-/-}$; OtxH embryos at 5 days post-fertilization.** Transverse sections of cerebellar areas from wild-type (**A**,**E**,**I**), OtxH (**B**,**F**,**J**), *fgf8*$^{-/-}$ (**C**,**G**,**K**) and *fgf8*$^{-/-}$; OtxH (**D**,**H**,**L**) embryos showing expression of *pax6* and *reelin* in granule cells (arrowheads in A-D and E-H, respectively) and zebrin in Purkinje cells (I-L). A total number of 10, 4 and 12 *fgf8*$^{-/-}$; OtxH embryos were analysed for *pax6*, *reelin* and zebrin staining, respectively. All the *fgf8*$^{-/-}$; OtxH embryos show the rescued phenotype. The count of zebrin-positive cells on vibratome 15 μm sections shows that a very small number of Purkinje cells are sometimes present [*fgf8*$^{-/-}$ embryos have 2.4±2.4 positive cells (*n*=4 embryos counted) whereas *fgf8*$^{-/-}$; OtxH embryos have 15±5 Purkinje cells per section (*n*=4)]. Cartoons represent transverse section through the cerebellum in wild type (**M**), OtxH (**N**), *fgf8*$^{-/-}$ (**O**) and *fgf8*$^{-/-}$; OtxH (**P**). Zebrin-positive cells are brow; *reelin* and *reelin+ pax6* granule cells are blue and green, respectively. Only a subset of reelin cells is positive for *pax6*. Granule cells are always missing in *fgf8*$^{-/-}$ (C,G). Ventral *reelin* staining corresponds to glial cells of the reticular formation, whereas proliferative regions of the midline at the level of the IVth ventricule and two lateral stripes of radial glia are *pax6* positive.

Nakayasu, 2001) in 5-day-old embryos. These two cell populations are expanded in OtxH embryos (Fig. 5B, *n*=30 embryos; Fig. 5F, *n*=16; Fig. 5J, *n*=36), and are absent in *fgf8*$^{-/-}$ (except the rare zebrin-positive cells in 10% of mutants, Fig. 5C, *n*=12; Fig. 5G, *n*=2, Fig. 5K, *n*=8). All *pax6*, *reelin* and zebrin-positive cell populations are rescued in *fgf8*$^{-/-}$; OtxH embryos (Fig. 5D, *n*=10; Fig. 5H, *n*=4; Fig. 5L, *n*=12).

Thus, upon reduction of Otx function, Fgf8 signalling is not required for the differentiation of the cerebellar granule and Purkinje cells. However, the rescued cerebellar region is significantly smaller than wild type. Cell death and proliferation were therefore carefully analysed (Fig. 6). Apoptosis, revealed by Acridine Orange staining was enhanced in double-mutant embryos from the 20-somite stage onwards, while no significant difference was observed in *ace* or OtxH embryos (Fig. 6A-D). Conversely, proliferation rate is decreased in prim-22 *ace* mutants and this diminution is not worsened by lack of Otx proteins (Fig. 6E-H). Thus, although Fgf activity is not required for cerebellar cell type specification, it is necessary for cell proliferation. Moreover, Otx and Fgf8 are both required for cell survival.

Finally, we analysed development of the r1-derived LC neurons. In *fgf8*$^{-/-}$; OtxH embryos [analysed at both the 15-somite stage (*n*=85) and prim-5 stage (*n*=52); data not shown], *phox2a* expression is never found, showing that LC fate requires *fgf8*, whether or not Otx function is lost.

## Fgf signalling is not instructive for the establishment of the r1 territory and differentiation of cerebellar identities

The presence of cerebellar differentiation in absence of Fgf8 in our OtxH morphants may mean that Fgf activity is required to maintain Otx expression away from r1 territory but is not necessary for any other step in cerebellar cell fate determination. However, a more trivial possibility is that some Fgf activity, carried out by other Fgfs in the MHB, may rescue cerebellar differentiation in the *fgf8*$^{-/-}$; OtxH embryos. To address this possibility, we measured Fgf activity in the MHB region. As the best indicator of Fgf gene activity is the expression of transcriptional targets, three of them were analysed in the four genetic contexts studied. As illustrated in Fig. 6I-P (and in Fig. S2 in the supplementary material), *erm, spry4* and *pea3* are highly expressed in wild-type MHB from bud stage onwards, but are never induced there in *fgf8*$^{-/-}$ and *fgf8*$^{-/-}$; OtxH embryos, indicating a complete absence of Fgf activity in this region. Finally, treating batches of *fgf8*$^{-/-}$ embryos with the Fgfr inhibitor SU5402 (Mohammadi et al., 1997) does not worsen the *fgf8*$^{-/-}$ midbrain phenotype (data not shown), further supporting a complete absence of Fgf signalling in the MHB of *fgf8*$^{-/-}$ mutants. The differentiation of cerebellar cell fates in our double loss-of-function embryos therefore occurs in absence of any Fgf activity in the MHB from at least bud stage onwards.

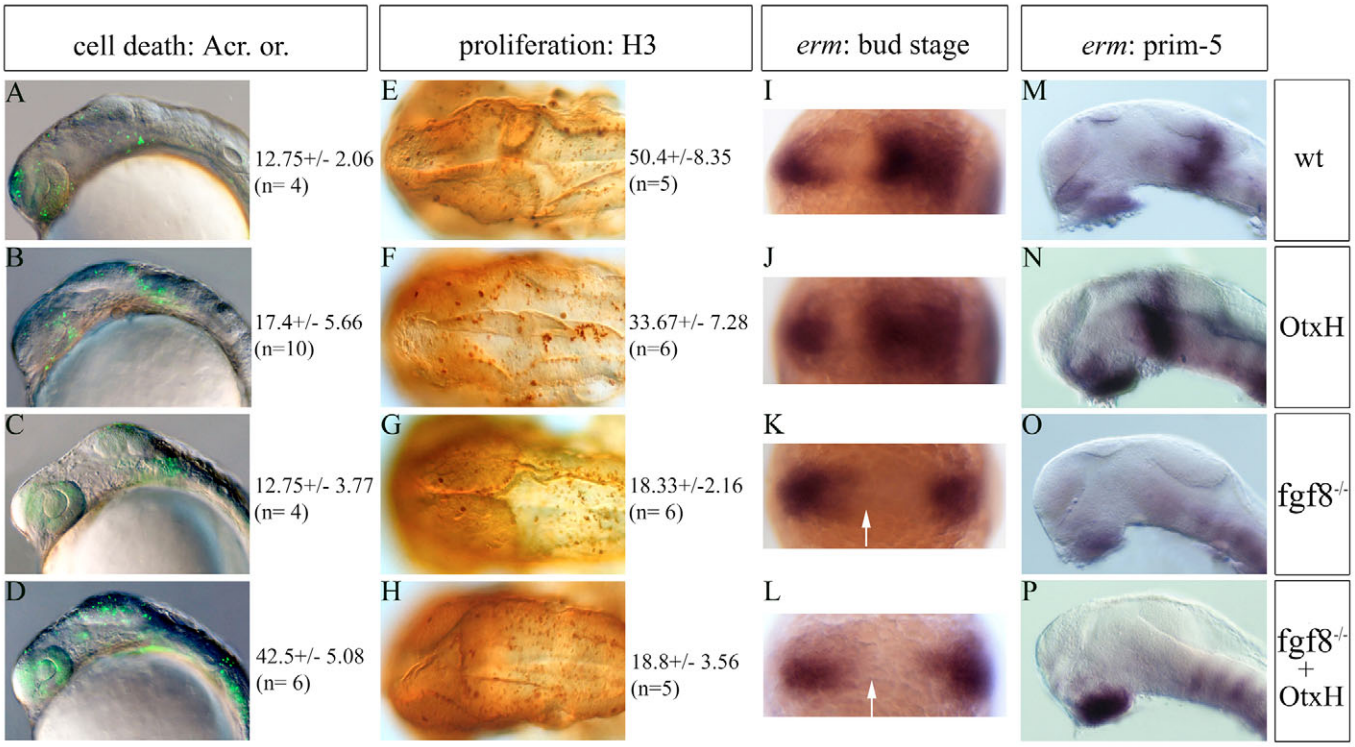


**Fig. 6. *fgf8*$^{-/-}$; OtxH embryos have a total lack of Fgf activity and show both a proliferation decrease and a cell death increase.** (**A-D**) Lateral view of 20-somite stage live embryos stained with Acridine Orange and cell quantification (*n*, numbers of quantified embryos). No change in cell death is observed in *fgf8*$^{-/-}$ mutant embryos (compare A with C), while OtxH shows an increase in dying cells (B), which is enhanced in *fgf8*$^{-/-}$; OtxH (D). These differences are more drastic at prim-22 stage (wild type: 18±4, *n*=3; OtxH 26±2, *n*=3; *fgf8*$^{-/-}$: 12±2, *n*=3; *fgf8*$^{-/-}$; OtxH: 44.33±6.03, *n*=3). (**E-H**) Dorsal views of prim-22 stage posterior brain immunostained with the H3 reveal a decrease in proliferation rate in *fgf8*$^{-/-}$ mutant (G), while lack of Otx in this context does not worsen the phenotype (H), see also quantifications. (**I-P**) *erm* expression pattern in the mid/hindbrain region of the genetic context studied at bud stage (dorsal view, I-L) and at prim-5 stage (lateral view, M-P). This Fgf transcriptional target is never induced in the presumptive mes/met (arrow) in *fgf8*$^{-/-}$ (K,O) or *fgf8*$^{-/-}$; OtxH (L,P) embryos.

To assess whether midbrain to r1 transformation can be observed in OtxH embryos totally depleted from earlier Fgf activity, we analysed the development of the mes/met area in OtxH embryos treated with SU5402 from the onset of gastrulation. Wild-type embryos treated with SU5402 present a marked reduction of *efnb2a* in presumptive r1, while, in SU treated OtxH morphant embryos, the anteroposterior extent of *efnb2a*-expressing r1 is similar to the one observed in untreated OtxH embryos (Fig. 7A-D). Early midbrain to r1 transformation is therefore taking place in the absence of Fgf activity in OtxH embryos. We then analysed *atoh1a* expression in the upper rhombic lip in wild-type and OtxH embryos, with or without SU5402 respectively. Results are summarized in Fig. S1L-O (see supplementary material). Inhibition of FgfR in wild-type embryos leads to the absence of prospective granule cells. By contrast, OtxH embryos subjected to the same treatment show formation of *atoh1a*-expressing granule cells. This experiment confirms that decreased level of Otx proteins allows for the development of cerebellar granule cell precursors in the complete absence of Fgf activity.

## Gbx2 is not strictly required for cerebellar differentiation

In higher vertebrates, two major transcription factors establish the presumptive mesencephalon and metencephalon [respectively, Otx2 (Martinez-Barbera et al., 2001) and Gbx2 (Wassarman et al., 1997)].

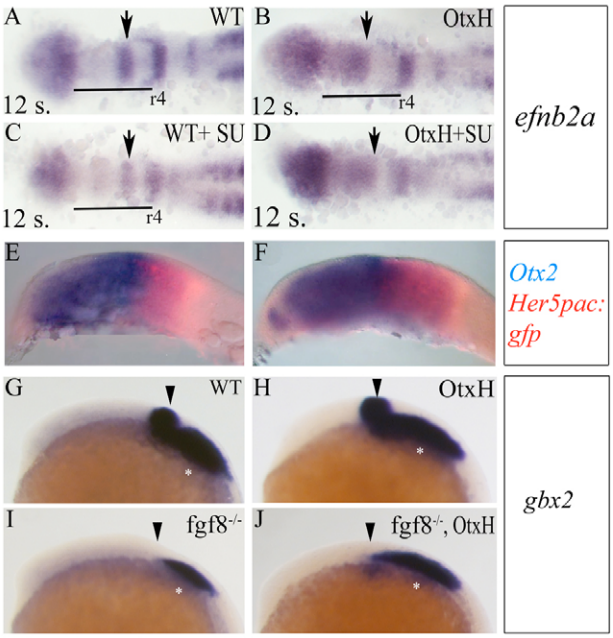


**Fig. 7. Early r1 rescue in *fgf8*$^{-/-}$; OtxH embryos is independent of *gbx2*.** Dorsal views, anterior towards the left showing *efnb2a* expression in wild-type (**A**, *n*=5/5), OtxH (**B**, *n*=8/9), wild type+SU5402 (**C**, *n*=12/15) or OtxH+SU5402 (**D**, *n*=18/23) embryos. The anteroposterior extent of r1 territory (arrows) is the same in OtxH with or without SU5402. Conversely, SU5402 treatment affects r5-7 development as previously reported (Maves et al., 2002). (**E**,**F**) Lateral views, anterior towards the left, of wild-type (E) and *fgf8*$^{-/-}$ (F) 8-somite stage embryos, showing double expression of *otx2* (blue) and her5pac:egfp (red). (**G-J**) Lateral views, anterior towards the left, showing *gbx2* expression in wild type (G), OtxH (H), *fgf8*$^{-/-}$ (I) or *fgf8*$^{-/-}$; OtxH (J). *gbx2* CNS expression is lost in *fgf8*$^{-/-}$ (arrows) and is not rescued in *fgf8*$^{-/-}$; OtxH (out of 21 *fgf8*$^{-/-}$; OtxH embryos, only five show a very faint rescue of Gbx2 expression in the very ventral metencephalic area). Asterisks indicate *gbx2* expression in the epibranchial placode precursors.

In *fgf8*$^{-/-}$ embryos, metencephalon precursors erroneously express *otx2*, indicating a posterior shift of mesencephalon identity (Jaszai et al., 2003; Tallafuss and Bally-Cuif, 2003), which we found can be revealed as early as the eight-somite stage (Fig. 7E,F). Complementarily, rostral expansion of r1 territory in OtxH embryos also starts at early somitogenesis, which may possibly be induced by rostral expansion of Gbx2 expression. Testing this possibility, we found that formation of cerebellar tissue, in *fgf8*$^{-/-}$; OtxH embryos, occurs independently of presence of *gbx2* (arrows in Fig. 7G-J). Zebrafish *gbx1* and *gbx2* are sequentially expressed in r1; and it has been suggested that *gbx1* is the functional homologue of mouse Gbx2 (Rhinn et al., 2003). Zebrafish *gbx1* expression is switched off in the wild-type mes/met area at a stage preceding the onset of mis-patterning in OtxH embryos and is not altered in these embryos, precluding its involvement in the process studied here. Conversely, *gbx2* expression is expanded anteriorly in OtxH embryos, as expected following the anterior shift of the r1 territory. As previously reported in fish and mouse (Chi et al., 2003; Rhinn et al., 2003), we confirm that maintenance of *gbx2* expression is dependent upon Fgf activity. However, we observed that loss of Otx function in *fgf8*$^{-/-}$; OtxH embryos is unable to rescue *gbx2* expression (Fig. 7J). As these embryos develop cerebellar fates, this implies that from the six-somite stage onwards, *gbx2* is not required for cerebellum cell differentiation in the absence of Otx function. Supporting this, a mouse conditional knockout showed that cerebellum still develops after *gbx2* inactivation at 9.5 dpc (Li et al., 2002).

## DISCUSSION

We show that, in zebrafish, double lack of *otx1-like* and *otx2* function leads to the transformation of the midbrain territory into an expanded presumptive r1, characterized by an enlarged cerebellum, highly reminiscent of the phenotype of *Otx2*$^{+/-}$; *Otx1*$^{-/-}$ mouse embryos (Acampora et al., 1997; Suda et al., 1997). It is worth noting that OtxH zebrafish embryos, like *Otx2*$^{+/-}$; *Otx1*$^{-/-}$ mice, present a transformation of retinal pigment epithelium into an enlarged retina (Fig. 2C and data not shown) (Martinez-Morales et al., 2001). This confirms a conserved role of Otx homeobox proteins across different species.

Taking advantage of these results, we analysed, for the first time, the mes-metencephalic phenotype of embryos lacking both Otx and Fgf functions in the MHB. We show that these embryos develop r1 identity and differentiate several cerebellar cell fates but lack the locus coeruleus. Thus, early r1 territory and cerebellar cell identities are induced in absence of Fgf signalling as long as Otx function is also removed. Our results therefore challenge the current model in which Fgf activity from the IsO is providing the information required for induction of cerebellar cell identities. If Fgf signalling is not instructive for cerebellar identity; it is, however, required to maintain the r1 compartment free of Otx protein, stabilizing compartment boundaries; it is also required not only for the proliferation of cerebellar precursor cells but also for cerebellar dorsoventral organisation and dorsal fusion.

## Cerebellar identities can differentiate in the absence of Fgf signalling activity

Fgf8 gain- and loss-of-function (Sato et al., 2001; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005; Trokovic et al., 2003) have led to a prevalent model in which Fgf8 signalling instructs cerebellar development. More recently, studies in fish, chick and ascidians propose a primary role for Fgf8 in repressing Otx expression, in vertebrates Otx2 in turn represses r1 and cerebellar identity (Hudson et al., 2003; Jaszai et al., 2003; Martinez et al., 1999;

Sato et al., 2001; Sato and Nakamura, 2004; Suzuki-Hirano et al., 2005). A mouse Fgf8 conditional null mutation producing a truncated protein similar to the one present in zebrafish *ace* has been reported (Chi et al., 2003). This shortened protein is also completely inactive in mouse, and embryos that carry this conditional knockout allele (which removes Fgf8 function inside the *En1*-expressing territory) show, like their zebrafish counterparts, a posterior shift of *otx2* expression at the 12-somite stage (Chi et al., 2003; Jaszai et al., 2003).

Here, we report that Fgf signalling is dispensable for cerebellar cell differentiation in the absence of Otx function. As we argue above, Otx2 is never expressed in presumptive r1. In chick and mouse, the posterior boundary of *otx2* expression defines the caudal limit of the presumptive midbrain and, hence, the anterior limit of the cerebellar anlage (Millet et al., 1996; Zervas et al., 2004). In fish too, three arguments strongly suggest that *otx2* is never expressed in prospective r1 territory. First, the posterior limit of *otx2* expression at late gastrulation is always anterior to that of *her5*, which maps to the posterior part of r1 (Tallafuss and Bally-Cuif, 2003; Tallafuss et al., 2001). Second, the gap between the posterior boundary of *otx2* expression and the anterior limit of the *hoxb1b* domain (earliest marker defining r3/r4 boundary) (Prince et al., 1998) is constant between 60% epiboly and the end of gastrulation (see Fig. S1 in the supplementary material). And third, *gbx1* expression only overlaps with o*tx2* at 60% epiboly, well before both the sharpening of the r1 boundary and the onset of *fgf8* expression in r4 (Reifers et al., 1998; Rhinn et al., 2003). Finally, the onset of *fgf8* expression in r1 is taking place during late gastrulation, long after the formation of the Otx expression domain in fish (Maves et al., 2002; Reifers et al., 1998; Rhinn et al., 2003) and mouse (Martinez-Barbera et al., 2001; Garda et al., 2001). All together, the existing data all strongly suggest that Fgf8 has no role in defining *otx2* posterior boundary initially but maintains it during somitogenesis. Hence, we propose that the function of Fgf signalling from the IsO is neither to induce r1 nor cerebellar cell identities but to prevent a later propagation of Otx expression inside the r1 territory, thereby maintaining a set of developmental decisions required to maintain r1, and preventing repression of cerebellar identities by Otx.

If it is not Fgf, which is the key molecule responsible for the switch to a cerebellar fate? Targeted mutations of either *otx2* or *hoxa2* resulted in either a rostral or a caudal expansion of the cerebellum, respectively (Acampora et al., 1997; Gavalas et al., 1997). Moreover, *hoxa2* mis-expression experiments in r1 showed cell-autonomous inhibition of granule cell fate (Eddison et al., 2004). Therefore, in Fgf misexpression experiments, the formation of an ectopic cerebellum would rather occur by local downregulation of the cerebellum repressors *otx2* and/or *hoxa2* (Irving and Mason, 1999; Martinez et al., 1999), rather than by direct induction. A member of the *iroquois* homeodomain gene family, *irx2* has been recently suggested as a specific inducer of chick cerebellar identity downstream of Fgf8 signal (Matsumoto et al., 2004). Exclusively in presence of Fgf activity, activated phosphorylated Irx2 forces the rostral hindbrain towards cerebellar fate. Although not discussed by the authors, the possibility of *irx2* attributes cerebellar identity independently from any Fgf activity cannot be excluded. Indeed, *iro1b* and *iro2a* are both expressed in r1 during early to mid-somitogenesis (Lecaudey et al., 2005). In our four conditions, both genes are still expressed in r1 of *fgf8*$^{-/-}$; OtxH embryos, showing that *iro1b* and *iro2a* are responding like other r1 markers studied (see Fig. S3 in the supplementary material). However, presence of these genes transcripts in both the mesencephalic and metencephalic compartment confirms that any r1-specific function of Iroquois should be studied at the protein level.

## Fgf8 is directly required for proliferation and morphogenesis of the cerebellum and specification of the locus coeruleus

There are great variations in cerebellum morphology among teleosts and the zebrafish cerebellum (mainly lateral and vestibular) is one of the simplest. The corpus cerebelli and valvula cerebelli are thought to be related to the mouse vermis and flocculi, respectively; and the corpus cerebelli, which received inputs from the vestibular apparatus, is believed to be the most primitive part of the cerebellum (Wullimann et al., 1996).

In our experiments, lack of Otx function affects the dorsal midline fusion that normally occurs during the early stage of cerebellar development. Based on an unexpected increase in isthmic structure in *En1*$^{+/Otx2LacZ}$ mouse embryos, it has been suggested that *otx2*-positive cells are crossing the *otx2* boundary to form a substratum region (called velum) required for midline fusion (Louvi et al., 2003). Even if, in zebrafish, the valvula cerebelli forms directly adjacent to the mesencephalon – without the presence of a recognizable velum – our results are compatible with a function of Otx in cerebellar dorsal fusion. Indeed, the absence of fusion in OtxH embryos suggests that Otx-expressing cells may be involved in midline fusion in fish.

This absence of fusion is exacerbated in absence of Fgf signalling. Phenotypic analysis of *fgf8*$^{-/-}$; OtxH zebrafish embryos shed some light on the Fgf function in cerebellar morphogenesis. *fgf8*$^{-/-}$; OtxH cerebellar tissue undergoes a severe lateral displacement. In particular, the granule cells of the eminentia granularis normally spread dorsoventrally in wild-type and OtxH embryos (Fig. 5A,B,E,F) but are only seen in the ventral area in *fgf8*$^{-/-}$; OtxH embryos (arrowheads in Fig. 5D,H). Finally, in *fgf8*$^{-/-}$; OtxH, the Purkinje cells loose their layered organisation and the ventral axonal projections are much reduced. Such projection has not yet been described in zebrafish and the only ventral projection described in adult teleost is thought to come from a specific subpopulation of caudal lobe Purkinje cells (Lannoo et al., 1991). Absence or disturbed axonal projections in the *fgf8*$^{-/-}$; OtxH cerebellum could therefore be due to either a defect in pathfinding, a delay in neuronal maturation or loss of a specific subpopulation of the Purkinje cell population. We therefore conclude that Otx activity is primarily required for cerebellar fusion, while Fgf8 activity is necessary for cerebellum folding and cellular organisation (but not differentiation) of the cerebellum.

The mechanisms that underlie cerebellar dorsoventral organisation and late cerebellar morphogenesis are also starting to be uncovered in mouse. Recent mouse studies reveal the importance of Hedgehog signalling for the cerebellar morphogenesis and its dorsoventral organisation (Sotelo, 2004; Wechsler-Reya and Scott, 1999). Our next challenge is therefore to understand how Fgf and Hh signals coordinate these events and what the downstream effectors are that they regulate in these processes.

Finally, our findings confirm the central role of Fgf in proliferation. In mouse, mis-expression of *fgf8* under the *wnt1* promoter revealed its mitogenic activity on mesencephalic cells (Lee et al., 1997) and partial *fgf8* lack of function reduces proliferation of the medial cerebellar anlage (Xu et al., 2000). This effect on proliferation is detected from prim-22 in *fgf8*$^{-/-}$ embryos, and reducing Otx levels in this context does not affect the proliferation rate, indicating that Otx is not involved in this process.

Conversely, cell death is not more frequent in absence of Fgf function, although the dying cells are more often found at the roof plate of the enlarged midbrain. By contrast, cell death is increased in OtxH mes/met area starting at 20-somite stage, most probably

owing to the lack of Wnt1 shown to be crucial for cell survival. This phenotype is exacerbated in absence of Fgf, probably revealing cooperation between these two signals. This result explains the reduced size (52% the size of OtxH; Fig. 4B,D) of the rescued granule cells population in *fgf8*$^{-/-}$; OtxH. At earlier stages, the overall size of the Her5-GFP territory is not dramatically different in double mutant embryos, neither is the expanded *efnb2a* territory in the SU-treated morphants, showing that there is no significant apoptosis during the first half of somitogenesis. Together, reduced proliferation and increased cell death explain the relatively variable number of rescued differentiated cells observed at 5 dpf in *fgf8*$^{-/-}$; OtxH.

Our study supports previous studies (Guo et al., 1999; Lam, 2003) showing that the IsO Fgf signalling is strictly required for the induction and/or early survival of LC tyrosin hydroxylase-positive precursors. Our data also indicate that loss of Otx function is not able to rescue LC neurons in *fgf8*$^{-/-}$mutant. This result suggests that, together with Bmp, Fgf is directly required to induce LC identity.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/10/1891/DC1

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