# Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by Wnt8 signaling

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## Summary

**The organizing center located at the midbrain-hindbrain boundary (MHB) patterns the midbrain and hindbrain primordia of the neural plate. Studies in several vertebrates showed that the interface between cells expressing Otx and Gbx transcription factors marks the location in the neural plate where the organizer forms, but it is unclear how this location is set up. Using mutant analyses and shield ablation experiments in zebrafish, we find that axial mesendoderm, as a candidate tissue, has only a minor role in positioning the MHB. Instead, the blastoderm margin of the gastrula embryo acts as a source of signal(s) involved in this process. We demonstrate that positioning of the MHB organizer is tightly linked to overall neuroectodermal posteriorization, and specifically depends on Wnt8 signaling emanating from lateral mesendodermal precursors. Wnt8 is required for the initial subdivision of the neuroectoderm, including onset of posterior *gbx1* expression and establishment of the posterior border of *otx2* expression. Cell transplantation experiments further show that Wnt8 signaling acts directly and non-cell-autonomously. Consistent with these findings, a GFP-Wnt8 fusion protein travels from donor cells through early neural plate tissue. Our findings argue that graded Wnt8 activity mediates overall neuroectodermal posteriorization and thus determines the location of the MHB organizer.**

Key words: Midbrain-hindbrain boundary, MHB/isthmic organizer, Posteriorization, Zebrafish, Neural plate, Morphogen, *wnt8, gbx1, otx2, gbx2, nodal, fgf*

## Introduction

The initial subdivision of the neural plate is the first step towards generating cellular diversity in the vertebrate brain. A key question is how this initial subdivision is achieved along the anteroposterior (AP) axis. In the two-step 'activation-transformation' model, an activating signal induces the ectoderm to become anterior neural tissue. Inhibition of bone morphogenic protein (Bmp) activity by factors released from the organizer like chordin, noggin or follistatin is thought to be crucial for this step (Wilson and Hemmati-Brivanlou, 1997). A second, transforming signal is thought to convert part of the neuroectoderm into a more posterior identity (transformation, or 'posteriorization'). Posteriorization has been difficult to understand because multiple signaling pathways can exert posteriorizing effects. Candidate molecules for posteriorizing signals are fibroblast growth factors (Fgfs), Wnt proteins and retinoic acid, but which signaling protein(s) are acting directly on target cells has been difficult to resolve (Gamse and Sive, 2000).

By the end of gastrulation, cell-type diversity of the neural plate is further refined through activities of local organizing centers. The midbrain-hindbrain boundary (MHB) organizer mediates positional information in the neuroectoderm via secreted molecules such as Fgf8 or Wnt1. Mutant analyses in mouse and zebrafish identified several genes involved in MHB development, e.g. *Wnt1*, *Pax2* and *Fgf8*, which are involved in the formation and organizing activity of the MHB (for reviews, see Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). Restricted gene expression in the zebrafish neural plate around the MHB is first observed during gastrulation stages (Lun and Brand, 1998; Reifers et al., 1998; Rhinn et al., 2003), and is prefigured by the interface between anterior *otx2*- and posterior *gbx1*-expressing cells. As in other vertebrates, this precise alignment suggests that forming the *otx2/gbx1* interface is a crucial step for positioning the MHB in the neural plate. Indeed, *Otx* and *Gbx* loss- and gain-of-function experiments in different species support the notion that these genes are required to correctly position the MHB in the neural plate (Wassarman et al., 1997; Rhinn et al., 1998; Rhinn et al., 2004; Acampora et al., 1998; Broccoli et al., 1999; Millet et al., 1999; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Kikuta et al., 2003) (M.R. and M.B., unpublished).

These findings raise the issue of how the interface itself is positioned in the early neural plate. Here, we study how the *otx2/gbx1* interface is set up in the zebrafish neuroectoderm. Regionally restricted *otx2* and *gbx1* expression occurs very early during gastrulation stages, and could be linked to the formation of axial mesendoderm. Conflicting results have been obtained concerning the involvement of the axial

mesendoderm in the AP patterning of the CNS. Studies in *Xenopus* have indicated that induction of AP neural patterning might involve signals from the involuted dorsal mesoderm to the overlying ectoderm (Ruiz i Altaba and Jessel, 1993). Work in several species suggests that signals from the anterior mesendoderm or notochord might regulate the expression of *Engrailed* genes in the neural plate (Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997; Shamim et al., 1999). However, these findings are inconsistent with node removal experiments, which reveal correct AP patterning, including *En2* expression, in the absence of axial mesendoderm (Darnell et al., 1992), and a role in refining rostrocaudal pattern has been suggested instead (Rowan et al., 1999; Camus et al., 2000). Moreover, in mouse or in zebrafish that lack the notochord, a well-formed neuraxis develops (Ang and Rossant, 1994; Weinstein et al., 1994; Klingensmith et al., 1999; Shih and Fraser, 1996; Saude et al., 2000). To address a possible (even transient) role, we examine the role of axial mesendoderm in positioning the MHB at the time it is first formed in zebrafish. We find that axial mesendoderm is not required for AP positioning of the MHB, but that the likely source of the signal is the blastoderm margin (non-axial mesendoderm). Thus, MHB positioning is related to the more general problem of posteriorization of the neuroectoderm (Woo and Fraser, 1997). Through blocking reception for individual signaling pathways, we identify Wnt signaling, and particularly Wnt8, as being required for initial establishment of *otx2* and *gbx1* territories, and present evidence that Wnt8 acts directly. We suggest that initial positioning of the MHB organizer is a direct consequence of overall posteriorization of the neuroectoderm, and that Wnt8 protein produced by lateral mesendodermal precursors is necessary to mediate this process directly.

## Materials and methods

### Strains and developmental conditions

The *flh* allele used is *flh*$^{tk241}$ (Odenthal et al., 1996) and the *ntl* allele used is *ntl*$^{tc41}$ (Odenthal et al., 1996). The *Dfw8* mutation (*Df(LG14)wnt8*$^{w8}$) was induced by γ irradiation (Lekven et al., 2001). Maternal-Zygotic *Oep* (MZ*oep*) fish where generated as described previously (Gritsman et al., 1999). Zebrafish were raised and kept under standard laboratory conditions at 28°C.

### Whole-mount in situ hybridization and antibody staining

In situ hybridization, probes and antibody staining against EN4D9 (Ntl) was performed as described previously (Brand et al., 1996; Reifers et al., 1998). Probes and wild-type expression patterns have been described elsewhere: *otx2* (Mercier et al., 1995), *wnt8* (Kelly et al., 1995), *pax2.1* (Krauss et al., 1991), *eng2* (Ekker et al., 1992; Fjose et al., 1988), *wnt1* (Kelly et al., 1993), *fgf8* (Reifers et al., 1998), and *gbx1* and *gbx2* (Rhinn et al., 2003).

### DNA construct and RNA synthesis

To generate the C-terminal Wnt8-GFP fusion protein, a *gfp*-coding sequence (Clontech) was inserted into pCS2+ vector (Rupp et al., 1994) via *Xba*I/*Sna*bI. *wnt8*-coding sequence was amplified by PCR and fused in frame into PCS2+/GFP vector. We synthesized capped mRNA as described previously (Reifers et al., 1998).

### RNA and morpholino injections

For RNA injections, embryos were dechorionated using pronase and injected at the one-cell stage. Morpholinos were designed and synthesized by Gene-Tools (Corvallis, OR). They were resuspended in sterile water, stored at –20°C as 10 mg/ml solutions and diluted before use to the appropriate concentration in water, 0.2% Phenol Red. For morpholino injections, embryos were dechorionated using pronase and injected in the yolk with 1-15 ng between the one- and four-cell stage.

### Transplantations

Donors embryos were injected with biotin-coupled tetramethylrhodaminedextran ($M_r$ 10,000, Molecular Probes D-1817) diluted in 0.25 M KCl. Transplantations of donor cells into host embryos were carried out at shield stage using trimmed borosilicate capillaries. Transplanted cells were then visualized by immunochemically staining using the Vectastain ABC system (VectorLabs) and the DAB system (Sigma).

### Pharmacological inhibition of Fgf signaling

To inhibit Fgfr activity, embryos were treated with Su5402 (Mohammadi et al., 1997) (Calbiochem) at 16 μM in E3 medium as described (Reifers et al., 2000).

## Results

### The blastoderm margin and not the axial mesendoderm is involved in positioning the *otx2/gbx1* interface

It has been postulated that, in vertebrate embryos, the axial mesendoderm is involved in specifying different AP segments of the neural axis (see Introduction). To address whether axial mesendoderm is required for correct AP positioning of the MHB primordium in the neural plate, we analyzed the expression of *otx2*, *gbx1* and the MHB markers *eng2* and *fgf8* in mutants for two transcription factors – *no tail* (*ntl/brachyury)* and *floating head* (*flh/Xnot)* – that lack notochord differentiation (Halpern et al., 1993; Schulte-Merker et al., 1994; Talbot et al., 1995). *otx2*, *gbx1*, *eng2* and *fgf8* are expressed at their correct AP position in *ntl* mutant embryos (Fig. 1E-H) and in *flh* mutant embryos (Fig. 1I-L) when compared with wild-type embryos (Fig. 1A-D).

Because axial mesendodermal progenitors are present in *ntl* and *flh* mutants, we removed these precursors by performing shield ablation experiments. Complete shield ablation was achieved by extirpating the shield plus adjacent territories of dorsal margin, as previously reported (Saude et al., 2000), and results in embryos missing the head and trunk notochord but forming a remarkably well-patterned nervous system (Shih and Fraser, 1996; Saude et al., 2000). The complete absence of dorsal mesendoderm in shield-ablated embryos was confirmed by the absence of axial Ntl staining (Fig. 1M-P). In such embryos, we find that the AP position of the MHB markers *otx2* (Fig. 1A,M; *n*=5/5), *gbx1* (Fig. 1B,N; *n*=3/3), *eng2* (Fig. 1C,O; *n*=3/3), and *fgf8* (Fig. 1D,P; *n*=4/4) is normal. However, in *ntl* mutants and shield-ablated embryos, *gbx1* expression was enhanced in the midline of the neural plate (Fig. 1, insets B′,F′,N′), and particularly in shield-ablated embryos, the midline gap between the neuroectodermal ‘wings’ of *fgf8* expression is more narrow (bracket, Fig. 1D,P), suggesting that signals repressing *gbx1* in the midline are lost. This finding suggests a possible role for axial mesendoderm in regulating *gbx1* expression in the midline neuroectoderm, but not in AP positioning. Together, these findings argue against a role of the

axial mesendoderm in positioning the early MHB primordium in the zebrafish neural plate.

Transplantation experiments in zebrafish suggested that the lateral blastoderm margin is involved in neural posteriorization at pre-gastrula stages (Woo and Fraser, 1997; Koshida et al., 1998). When cells from this region are transplanted into the animal pole of the embryo, hindbrain markers such as *krox20* and *hoxa1*, are induced in the host tissue, suggesting that secreted molecules are involved in this process (Woo and Fraser, 1997; Koshida et al., 1998; Momoi et al., 2003).

In order to analyze a possible involvement of the blastoderm margin in the establishment of the *otx2/gbx1* interface, we transplanted cells from the blastoderm margin situated at a defined angular distance from the shield (30-45°), to the animal pole, a region fated to become forebrain. Expression analysis shows that *otx2* is repressed (Fig. 2A,A′; *n*=50/50) (Koshida et al., 1998), whereas *gbx1* is induced ectopically (Fig. 2B,B′; *n*=25/40) around the transplanted clones as early as 2 hours after transplantation. The ability of the blastoderm margin to repress *otx2* and to induce *gbx1* strongly suggests that the signal for correct positioning of the *otx2* and/or the *gbx1* expression domain arises from the blastoderm margin.

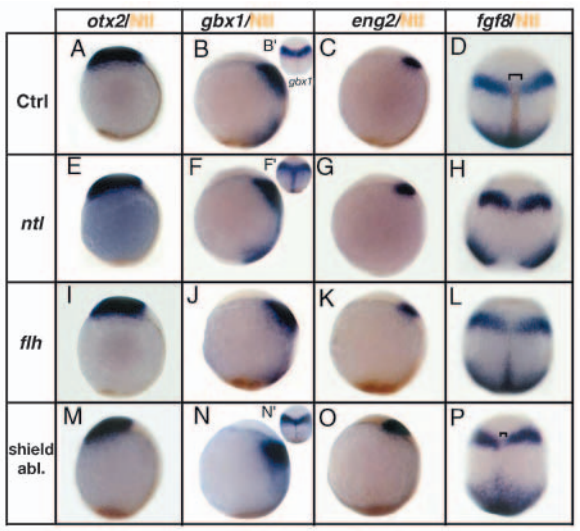



**Fig. 1.** Expression of MHB markers is normal in mutants with defects in notochord formation and in shield-ablated embryos. (A-D) In situ hybridization and Ntl immunostaining (brown) of control embryos, (E-H) *ntl* mutant embryos, (I-L) *flh* mutant embryos and (M-P) shield-ablated embryos at tailbud stage. Probes were applied: *otx2* (A,E,I,M), *gbx1* (B,F,J,N), *eng2* (C,G,K,O), *fgf8* (D,H,L,P). In B′, no Ntl immunostaining has been performed. No changes in the AP position of the different MHB markers analyzed is observed in *ntl* mutants, in *flh* mutants or in shield-ablated embryos when compared with wild-type embryos. (D,P) Brackets indicate the reduced midline width following shield ablation. (A-O) Lateral views, anterior upwards; (D,H,L,P,B′,F′,N′) dorsal views, anterior upwards.

## The establishment of the *gbx1* and *otx2* expression domains involves putative posteriorization signals

The results above indicate that a signal from the blastoderm margin is able to regulate *otx2* and *gbx1* expression. Several candidate signals are produced by cells at the blastoderm margin: retinoic acid, Fgfs, Nodals and Wnts. Analysis of *gbx1* and *otx2* expression in embryos treated with BMS493, a pan-retinoic acid receptor antagonist, shows no changes in initial positioning of *gbx1* and *otx2* expression domain (Grandel et al., 2002) (data not shown). By contrast, global disruption of all other pathways interferes with normal *gbx1* and *otx2* expression.

(1) *fgf3*, *fgf8* and *fgf24* are expressed in the margin during gastrulation (Fürthauer et al., 1997; Reifers et al., 1998; Raible and Brand, 2001; Draper et al., 2003). Indeed, *gbx1* and *otx2* expression is altered in wild-type embryos in which Fgf signaling has been blocked pharmacologically using Su5402 (Mohammadi et al., 1997), a potent inhibitor that blocks all Fgf receptor signaling (Fig. 2D,H). Su5402 treatment causes a posterior shift of *otx2* expression mediodorsally (Fig. 2D), probably reflecting the enhanced ventralization after loss of Fgf

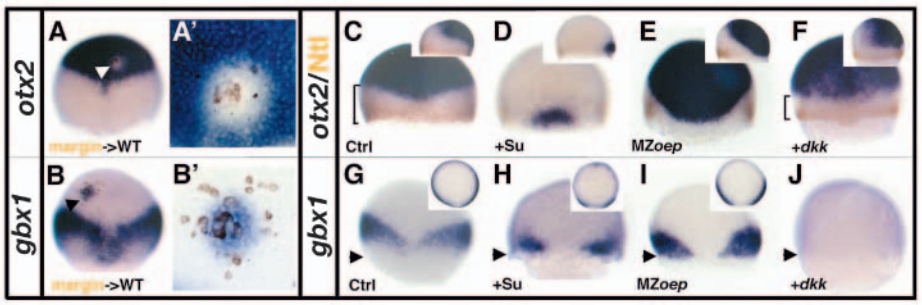



**Fig. 2.** The blastoderm margin is the source of signals important for *otx2* and *gbx1* expression. (A-B′) Lateral blastoderm margin transplantations into the animal pole of a wild-type host embryo. (A,B) Embryo containing cells from the blastoderm margin derived from injected wild-type embryos with a lineage tracer (brown). (A) The white arrowhead indicates a clone of non-*otx2*-expressing cells in the *otx2* domain of a transplanted embryo. (A′) Close-up of the transplanted cells. *otx2* is repressed around the transplanted cells. (B) The black arrowhead indicates a clone of *gbx1*-expressing cells in the *otx2* domain. (B′) Close-up of the transplanted cells. *gbx1* expression is induced around the transplanted cells. (C-J) *gbx1* and *otx2* expression in the absence of FGF, Nodal or Wnt signaling at 60%. (C) Control embryo stained for *otx2* and Ntl protein (brown). (D) In Su5402-treated embryos, *otx2* is strongly reduced and expands up to the margin at the level of the midline. (E) In MZ*oep* embryos, *otx2* expands up to the margin at the level of the midline. (F) In *dkk1*-injected embryos (100 pg), a posterior shift of *otx2* is visible laterally (compare brackets in C and F). (G) Control embryo stained for *gbx1*. *gbx1* is expressed dorsolaterally and absent in the margin ventrally in Su5402-treated embryos (H). (I) In MZ*oep* embryos, *gbx1* expands up to the margin. (J) *dkk1*-injected embryos (100 pg) do not express *gbx1*, showing the Wnt-dependent activation of *gbx1*. The black arrowheads indicate the margin. (C-J) Dorsal views, anterior upwards; (C,D,E,F) right corner, lateral views, dorsal towards the right; (G-I) right corner, animal pole views, dorsal downwards.

function (Fürthauer et al., 2004). In addition, ventral *gbx1* expression is strongly reduced in Su5402-treated embryos (Fig. 2H insets), presumably owing to the lack of induction of prospective vegetal neural fate (Kudoh et al., 2004; Rentzsch et al., 2004).

(2) Nodal-related factors are required for mesendoderm induction, and also affect *gbx1* and *otx2* expression, as studied in Maternal-Zygotic *one-eyed-pinhead* (MZ*oep*) mutants that are unresponsive to Nodal signaling (Gritsman et al., 1999; Schier, 2001). MZ*oep* mutants show an expansion of *gbx1* expression up to the margin (Fig. 2I, arrowhead) and a posterior shift of *otx2* expression mediodorsally (Fig. 2E), presumably owing to the involvement of Nodal in the specification of dorsal mesendoderm.

(3) *dickkopf-1* (*dkk1*) is a secreted inhibitor of Wnt signaling (Glinka et al., 1998; Hashimoto et al., 2000). *dkk1* mRNA injection causes complete absence of *gbx1* expression at 60% of epiboly (Fig. 2J). However, by 80% of epiboly, *gbx1* expression recovers to nearly its normal size, although its location shifts more posteriorly (data not shown). Simultaneously, a posterior shift of *otx2* expression is observed in *dkk1*-injected embryos, severely reducing the gap between the posterior border of *otx2* and the blastoderm margin (Fig. 2F). In summary, establishment of *gbx1* and *otx2* expression is strongly affected when Nodal, Fgf or Wnt signals are inhibited in whole embryos, but these experiments do not distinguish direct from indirect action of these pathways.

## Wnt signaling directly defines the posterior border of *otx2*

To test how direct the action of these factors is, we examined whether clones of cells that are 'blind' to Fgf, Nodal or Wnt proteins – owing to disrupted signal reception – can respond to AP positional information in an otherwise wild-type environment. A dominant-negative *Xenopus* Fgf receptor, XFD (Amaya et al., 1991) makes cells unresponsive to Fgf signaling when overexpressed. *XFD* mRNA-injected donor embryos show the same defects in *otx2* and *gbx1* expression as Su5402-treated embryos and fail to activate the Fgf8-target gene *sprouty4* (Fürthauer et al., 2001) (Fig. 3O,P). We transplanted cells from *XFD*-injected donors into wild-type host embryos and monitored their fate. The transplanted cells showed *otx2* and *gbx1* expression appropriate to their location (Fig. 3A-D; *otx2*, 40/40; *gbx1*, 30/30) [for *otx2* see also Koshida et al. (Koshida et al., 1998)], demonstrating that Fgf signaling is not directly involved in defining the *otx2*/*gbx1* interface. Likewise, MZ*oep* donor-derived cells that are unresponsive to Nodal signaling show *otx2*/*gbx1* expression appropriate to their location (Fig. 3E-H; *otx2*, 70/70; *gbx1*, 60/60), showing that Nodals are also not directly involved.

By strong contrast, blocking Wnt-signal reception in receiving neuroectodermal cells by *ΔNTcf3* mRNA injection provides evidence for a direct involvement of Wnt signaling. *ΔNTcf3* encodes a N-terminal deletion of *Tcf3* that lacks the β-catenin-binding domains, but retaining its repressor activity, and thus renders overexpressing cells unresponsive to Wnt signaling (Molenaar et al., 1996). Embryos injected with *ΔNTcf3* mRNA do not express *gbx1* at 60% of epiboly (data not shown) like *dkk1*-injected embryos (Fig. 2J), and expression is strongly diminished of *eng2,* a known β-catenin/Tcf signaling target (Kim et al., 2000) (Fig. 3Q,R). We

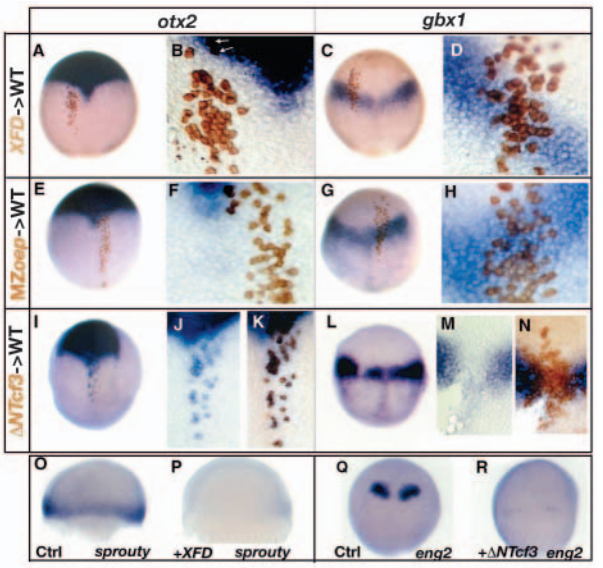



**Fig. 3.** Role of FGF, Nodal and Wnt signals in defining the *otx2* and the *gbx1*-expression domain. (A-D) Embryo containing cells derived from injected embryos with a lineage tracer (brown) and *XFD* RNA (200 pg) stained for *otx2* (A,B) or for *gbx1* expression (C,D) at 90%. (B,D) Close-up of transplanted cells. *XFD*-transplanted cells (blind for FGF signaling) strictly follow the host *otx2* expression domain or *gbx1* expression domain. (E-H) Embryos containing cells derived from MZ*oep*-injected embryos with a lineage tracer (brown) stained for *otx2* (E,F) or for *gbx1* expression (G,H) at 90%. (F,H) Close-up of transplanted cells. MZ*oep* cells (blind for Nodal signaling) strictly follow the host *otx2* expression domain or *gbx1* expression domain. (I-N) Embryos containing cells derived from injected embryos with a lineage tracer (brown) and *ΔNTcf3* RNA (400 pg). (I) Transplanted embryo stained for *otx2*. (J) Close-up of cells ectopically expressing *otx2* followed by (K) staining the donor cells (brown). (L) Transplanted embryo stained for *gbx1*. (M) Close-up of the gap in the *gbx1*-expression domain followed by (N) staining the donor cells (brown). The *ΔNTcf3*-transplanted cells (blind for Wnt signaling) express *otx2* ectopically and do not express *gbx1*. (O) Control embryo at 60% stained for *sprouty4*; (P) XFD-injected embryos do not express *sprouty4*, an FGF target gene. (Q) Control embryo at tailbud stained for *eng2*. (R) In *ΔNTcf3*-injected embryos, *eng2* expression is strongly diminished. (A-R) Dorsal views, anterior upwards.

transplanted cells from *ΔNTcf3*-injected embryos into wild-type embryos and monitored the fate of the transplanted cells. Importantly, and in contrast to cells blind to Nodals or FGFs, cells blind for Wnt show ectopic *otx2* expression outside the endogenous *otx2* expression domain (Fig. 3I-K; 34/34). Similarly, when located within in the *gbx1* domain, these cells do not express *gbx1* (Fig. 3L-N; 12/20). Together these data argue that the requirement of Fgf and Nodal signaling is indirect, whereas Wnt signaling may act directly to position *otx2* and *gbx1* expression in the neural plate.

## Expression of *gbx1* and *otx2* is regulated by Wnt8

Among several Wnt genes expressed in the blastoderm margin, *wnt8* shows the closest correlation in spatiotemporal expression to the expected signal with an onset in the blastoderm margin at 50% of epiboly and exclusion from the shield (Kelly et al., 1995; Lekven et al., 2001; Erter et al., 2001). To test a possible role of *wnt8* for activation of *gbx1*

expression, we expressed *wnt8* mRNA in all cells by injecting into one-cell stage zebrafish embryos. Depending on the amount of injected *wnt8* mRNA, different levels of *gbx1* expression are induced. Compared with the normally posteriorly restricted domain of *gbx1* expression, low doses (1-5 pg) enlarge the *gbx1* domain, but leave the animal pole area free of *gbx1* expression (Fig. 4A-B). Intermediate doses of *wnt8* mRNA (10-50 pg) induce high levels, whereas high doses (200-400 pg) induce lower levels of *gbx1* expression throughout the neuroectoderm (Fig. 4A,C,D). Conversely, *otx2* repression is apparent at low doses (1-5 pg) but more complete at intermediate (10-50 pg) and high doses (200-400 pg) of *wnt8* mRNA (Fig. 4G-J). In these experiments, we observed that *gbx1* can be induced both in prospective neural and non-neural ectoderm (Fig. 4A-D). We therefore asked if neural induction precedes *gbx1* induction by *wnt8* ectopic expression in prospective non-neural ectoderm, by investigating whether the non-neural markers *foxi1* and *p63*, or the pan-neural markers *sox31* and *zic2.2* (Kudoh et al., 2004; Rentzsch et al., 2004) are induced or suppressed, respectively. We observe that the non-neural markers tested are repressed, and that neural markers are expanded throughout the ectoderm (see Fig. S1 in supplementary material). This is also true when Wnt8 is expressed from transplanted clones of cells (see Fig. 6O-Q″). These findings suggest that *wnt8* gain-of-function is associated with induction of neural tissue. Further studies will need to address how direct this effect is and/or which other molecules may cooperate in promoting induction of prospective neural fate under these conditions (see also Baker et al., 1999). We also found that Wnt8 can act independently of Nodal signaling (Fig. 4F,L), but does require Fgf signaling for *gbx1* expansion, though not for *otx2* repression (Fig. 4E,K), probably reflecting the ventralization of the neuroectoderm following Fgf inhibition (Fürthauer et al., 2004). As expected, inhibition of Fgf signaling through Su5402, or of Nodal signaling in MZ*oep* embryos, eliminates or strongly diminishes *wnt8* mRNA expression dorsally (Fig. 4M-O).

### Loss of Wnt8 shifts the position of the MHB primordium posteriorly

A crucial test is to determine whether Wnt8 loss-of-function causes mispositioning of the *gbx1/otx2* interface. We extended previous *wnt8* loss-of-function studies (Erter et al., 2001; Lekven et al., 2001) to investigate the dependency of *gbx1* and *otx2* on Wnt8. We knocked-down *wnt8* either by injecting antisense morpholino oligonucleotides or by using *Df(LG14)wnt8*$^{w8}$ (*Df(wnt8)*) mutants, which carry a deletion of a region of linkage group 14 containing the *wnt8* locus and other genes (Lekven et al., 2001). In both loss-of-function situations, *gbx1* expression is absent from the ectoderm until 65-70% of epiboly (Fig. 5A-C). During later epiboly stages, *gbx1* expression initiates, but in an inappropriate, more posterior position at the margin itself, overlapping with the Ntl expression domain (Fig. 5D-F). Because at its very onset *gbx1* is expressed transiently at the margin (Rhinn et al., 2003), we suggest that in the absence of *wnt8* function, this earlier stage of *gbx1* expression may be retained. These observations suggest that Wnt8 is required for *gbx1* activation and positioning during early gastrulation. Furthermore, the requirement for Wnt8 diminishes during later gastrulation stages, suggesting that additional signal(s) are capable of activating *gbx1* expression at stages beyond 65% of epiboly.

In morphants and *Df(Wnt8)* mutants, the posterior border of *otx2* expression is shifted posteriorly at the ventrolateral level at all stages of development investigated (Fig. 5G-L, red arrowheads). This posterior shift is apparent already when *gbx1* and *otx2* still overlap (60% of epiboly), and the mutually repressive interactions between them are presumably not yet operating (Rhinn et al., 2003). This argues that at 60% of epiboly, Wnt8 also positions the posterior border of the *otx2* expression domain. Interestingly, however, even in the *Df(Wnt8)* mutants a small gap persists between the posterior border of the *otx2* and the Ntl expression domain (Fig. 5G-L), suggesting that additional factor(s) are involved in positioning the posterior border of *otx2*. These additional factor(s) are unlikely to be other redundant members of the Wnt family,

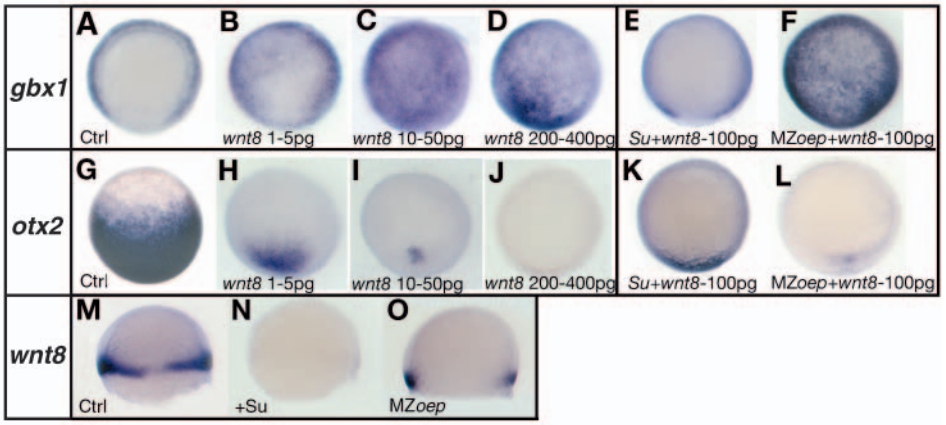



**Fig. 4.** *gbx1* and *otx2* expression in Wnt8 gain-of-function experiments. (A) Control embryo stained for *gbx1* at 60%. (B-D) Injected embryos with *wnt8* RNA 1-5 pg (B), 10-50 pg (C) and 200-400pg (D) stained for *gbx1*. Overexpression of *wnt8* induces ectopic *gbx1* expression throughout the epiblast in a dose-dependant way. Overexpression of *wnt8* (100 pg) does not induce ectopic *gbx1* in Su5402-treated embryos (E) but does so in MZ*oep* embryos (F). (G) Control embryo stained for *otx2* at 60%. (H-J) Injected embryos with *wnt8* RNA 1-5 pg (H), 10-50 pg (I) and 200-400 pg (J) stained for *otx2*. Overexpression of *wnt8* inhibits *otx2* expression. Overexpression of *wnt8* (100 pg) in Su5402-treated embryos (K) and in MZ*oep* embryos (L) inhibits *otx2* expression. (M-O) *wnt8* expression in wild type at 60% (M), in Su5402-treated embryos (N) and in MZ*oep* embryos (O). (A-L) Animal pole views. (M-O) Dorsal views, anterior upwards.

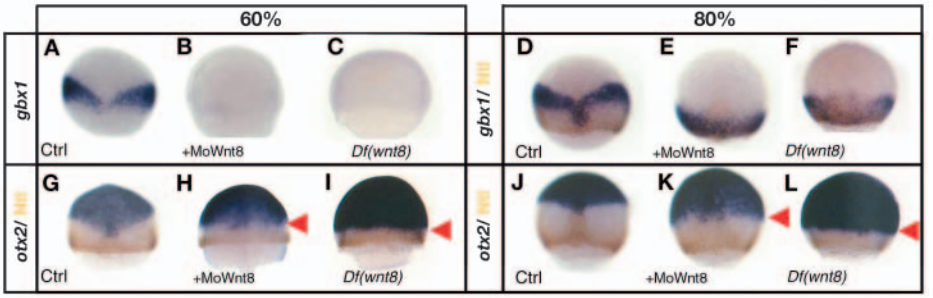



**Fig. 5.** *gbx1* and *otx2* expression in Wnt8 loss-of-function experiments. (A-C) Embryos stained for *gbx1* and (D-F) embryos stained for *gbx1* and Ntl protein (brown). (A-C) Control embryo (A), embryo injected with MoWnt8 (B) and *Df(Wnt8)* mutant embryos (C) at 60%. *gbx1* is not expressed at 60% in absence of Wnt8. (D-F) Control embryo (D), embryo injected with MoWnt8 (E) and *Df(Wnt8)* mutant embryo (F) at 80%. *gbx1* expression is observed at 80% onwards and overlaps partially with the Ntl domain. (G-L) Embryos stained for *otx2* and Ntl protein (brown). (G-I) Control embryos (G), embryo injected with MoWnt8 (H) and *Df(Wnt8)* mutant embryo (I) at 60%. (J-L) Control embryos (J), embryos injected with MoWnt8 (K) and *Df(Wnt8)* mutant embryo (L) at 80%. Laterally a posterior shift of the *otx2* expression domain is visible (red arrowheads) in the morphants and in the *Df(Wnt8)* mutant embryos. (A-L) Dorsal views, anterior upwards.

based on three pieces of evidence. First, injection of antisense morpholino oligonucleotides against *wnt3a* (Buckles et al., 2004), another Wnt gene expressed in the margin slightly later than *wnt8*, does not affect *otx2* positioning; and co-injection with the *wnt8* morpholino does not increase the extent of the *otx2* domain towards the margin (data not shown). Second, no other Wnt from the canonical pathway has been shown to be expressed in the correct time and place to have an effect on *otx2* and/or *gbx1*. Third, injection of *dkk,* as a general inhibitor of all Wnts signaling through the canonical pathway, does not generate a stronger phenotype than that observed in *wnt8* morphants or the *Df(Wnt8)* mutants (compare Fig. 2F with Fig. 5H,I), suggesting that no other Wnt molecules are involved.

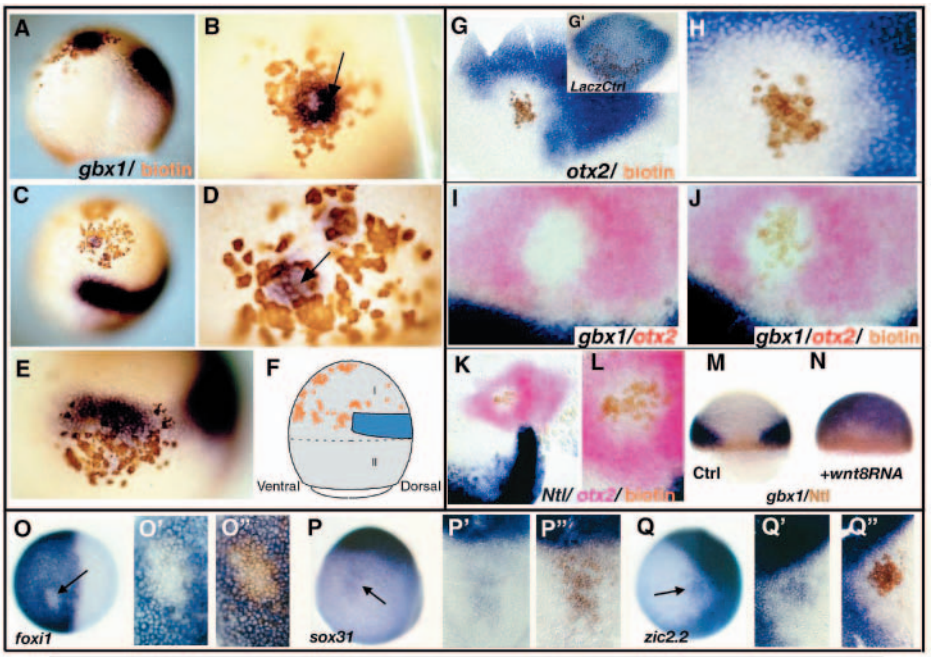
### Wnt8 can repress *otx2* expression independently of *gbx1* and mesendoderm

To reveal how direct the effect of Wnt8 on *gbx1* and *otx2* is, we transplanted *wnt8*-overexpressing cells into wild-type host embryos at pre-gastrula stages, and analyzed by in situ hybridization for induction of *gbx1* and repression of *otx2*. Host embryos carrying such clones showed ectopic *gbx1* expression (Fig. 6A-F,R), both in the transplanted cells and in the surrounding host tissue (Fig. 6B,D,E). Fig. 6F summarizes all locations in which Wnt8-expressing clones were able to induce *gbx1* expression in host cells. Although the Wnt8-expressing clones were distributed essentially randomly, it appears to be more 'difficult' to activate *gbx1* expression close to the margin (zone II in Fig. 6F), probably because the level of endogenous Wnt8 in zone II is too high to allow *gbx1* induction (akin to the suppression observed at high doses of injected *wnt8* in Fig. 4D). Similarly, fewer clones were found dorsoanteriorly, probably owing to the activity of extracellular Wnt inhibitors in the anterior neural plate (Wilson and Houart, 2004) (see below). Within the *otx2* domain, transplanted Wnt8-expressing cells were able to repress *otx2* expression in the host over about five cell diameters (Fig. 6G,H,R). This repression was never observed in control embryos transplanted with cells from donor embryos injected with *lacZ* mRNA (Fig. 6G′). Contrary to a previous report (Agathon et al., 2003), we never observed *wnt8* induction in neighboring non-transplanted host cells, arguing that *wnt8* expression does not 'self-induce' (see Fig. S2 in supplementary material). To further confirm this result, we showed that repression of *otx2* around a *wnt8*-expressing transplanted clone of cells does not require *wnt8* function in the host embryo, as tested in embryos homozygous for *Df(wnt8)* (see Fig. S2 in supplementary material).

Considering the importance of mutual repression between *otx2* and *gbx1* at later stages, we sought to investigate if the repression of *otx2* by Wnt8-expressing clones requires *gbx1* function. In embryos transplanted with *wnt8*-overexpressing cells we do not observe *gbx1* induction when *otx2* is suppressed around the transplanted clone (Fig. 6I,J), arguing that the repression of *otx2* by Wnt8 is not mediated through *gbx1*. Given the previously reported Wnt8-function in specifying ventrolateral mesodermal fate (Christian et al., 1991; Lekven et al., 2001), could Wnt8 indirectly alter *otx2* and *gbx1* expression through inducing mesoderm secondarily? Probably not, because *gbx1* expression is induced throughout the ectoderm in *wnt8*-mRNA injected embryos, whereas Ntl expression is only slightly broadened at the blastoderm margin (Fig. 6M,N) (Kelly et al., 1995). Moreover, *otx2* inhibition or *gbx1* induction is not linked with mesendoderm induction, as seen in double in situ hybridization on transplanted embryos with a *ntl* and an *otx2* probe (Fig. 6K,L,R), in which we never detected ectopic *ntl* expression around the transplanted clone. Therefore, *otx2* inhibition and *gbx1* activation around clones of Wnt8-expressing cells are more likely due to a direct effect of Wnt8.

### Wnt8 does not act via a relay mechanism to inhibit *otx2* expression

To test this notion further, we examined if Wnt8 directly inhibits *otx2* and activates *gbx1*, rather than doing so through other factors ('relay mechanism'), using three different approaches. First, we transplanted Wnt8-expressing cells ectopically into host ectoderm that cannot receive Wnt8 signaling, because they express *Fzb1-gpi* (Momoi et al., 2003), a competitive secreted inhibitor that binds Wnts and thus should block the effects of Wnt8 clones on *otx2* and *gbx1* expression if signaling is direct. The gpi anchor of Fzb1-gpi prevents Fzb1 from diffusing into the transplanted clone, and should thus allow them to produce Wnt8. In such embryos, *otx2* repression is limited to the transplanted cells, and is not




R

| Phenotype (%) | Wnt8-> WT | Wnt8-> WT only clones in area I counted |
|---|---|---|
| No *gbx1* expression | 80 (n=77) | 27 (n=19) |
| Ectopic *gbx1* expression | 20 (n=77) | 73 (n=19) |
| *otx2* repression | --- | 66 (n=99) |
| Ectopic *Ntl* expression | 0 (n=25) | 0 (n=20) |

**Fig. 6.** Wnt8 can induce *gbx1* and repress *otx2*. (A-E) Embryos containing cells derived from embryos injected with a lineage tracer (brown) and *wnt8* RNA (400 pg) stained for *gbx1* expression. (A) Lateral view and (B) close-up of the transplanted cells seen in A; the arrow indicates *gbx1* expression in the transplanted cells. (C) Lateral animal pole view of a transplanted embryo and (D) close-up of the transplanted cells. *gbx1* is induced in the host tissue but also in the transplanted cells (arrow). (E) Strong induction of *gbx1* expression in the host embryo at the AP level of the endogenous *gbx1* domain. (F) Summary of all transplanted cells clones that induced *gbx1* expression. The embryo can be subdivided into a *wnt8*-responsive part (I) and non-responsive part (II). (G) *wnt8*-overexpressing cells (brown) transplanted into wild-type embryos and stained for *otx2*. (H) Close-up of the transplanted cells. *otx2* is repressed in the transplanted cells and in the host cells. (G′) Control *lacZ*-overexpressing cells (brown) transplanted in wild-type embryos and stained for *otx2* expression. No repression in the transplanted cells and host cells is observed. (I) *wnt8*-overexpressing cells transplanted into wild-type embryos and stained for *otx2* (red) and *gbx1* expression (blue); (J) close-up of the transplanted cell after biotin staining (brown). No ectopic *gbx1* expression is seen in the transplanted area where *otx2* is repressed. (K) *wnt8*-overexpressing cells (brown) transplanted into wild-type embryos and stained for *otx2* (red) and *ntl* expression (blue); (L) close-up of the transplanted cells. No ectopic *ntl* expression is seen in the transplanted area where *otx2* is repressed. (M) Control embryo at 60% stained for *gbx1* (blue) and Ntl protein (brown). (N) Overexpression of *wnt8* induces ectopic *gbx1* throughout the epiblast and Ntl expression is expanded from three or four rows of cells. (O-Q″) *wnt8*-expressing clones correlate with loss of non-neural, and gain of neural marker induction. (O-O″) *foxi1* expression; (O) animal pole view. (P-P″) *sox31* expression, (Q-Q″) *zic2.2* expression. (P,Q) Lateral views. Close-up of the transplanted cells (O′,P′,Q′) before biotin staining and (O″,P″,Q″) after biotin staining. *foxi1* repression occurs in the transplanted cells and *sox31* and *zic2.2* are induced. (R) Observed ectopic induction of *gbx1,* repression of *otx2* and induction of Ntl when *wnt8*-overexpressing cells were transplanted into wild-type host embryos. In the last column, only cell clones within the endogenous *gbx1* domain and the animal pole, the area I (see Fig. 6F), are scored. n, number of transplanted embryos.

observed in surrounding host cells (Fig. 7A-A″, 10/15). Likewise, *gbx1* is induced only in transplanted cells and not in host cells (Fig. 7B-B″, 75/80). In a few (5/80) cases, *gbx1* is expressed in one-two cells around the transplanted clone, probably reflecting a slight variability in the Fzb1-gpi inhibition.

Second, we transplanted cells in which the Wnt pathway is activated in a ligand-independent way, and analyzed if these clones phenocopy the effects of a *wnt8*-expressing clone. In the absence of Wnt signaling, Glycogen synthase kinase-3β (Gsk-3β) is part of a multiprotein complex containing Axin, APC and β-catenin that phosphorylates β-catenin to target it for ubiquitination and subsequent degradation. A lysine-to-arginine exchange in the ATP-binding domain generates a ‘kinase-dead’ mutant of *Xgsk3,* resulting in a dominant-negative variant that suppresses Wnt signal transduction (Xgsk-3K→R) (Pierce and Kimelman, 1996). Consistent with the involvement of Wnt signal transduction, embryos injected with *Xgsk-3K→R* mRNA do not express *otx2* at 60% of epiboly (Fig. 7E,F) and show ectopic *gbx1* expression (Fig. 7G,H) as do *wnt8*-injected embryos (Fig. 4A-D,G-J). If secondary secreted molecules were involved, *otx2* should be repressed not only in the transplanted cells, but also in the cells around a cell clone expressing *Xgsk-3K→R*. Alternatively, if Wnt8 directly represses *otx2*, the repression should be observed cell-autonomously, i.e. restricted to the transplanted

cells. We find that *otx2* repression and *gbx1* induction is now limited to the transplanted cells (Fig. 7C-C″, *otx2*, 24/25; Fig. 7D-D″, *gbx1*, 18/20). Importantly, and in contrast to the transplantation of *wnt8*-injected cells, we now observe gbx1 induction also more frequently in dorsal positions (Fig. 7D). In the transplanted cells expressing ectopic *Xgsk-3K→R* mRNA, the Wnt pathway is thought to be activated independently of Wnt molecules. Secreted Wnt-inhibitors from the animal pole and acting dorsally (Wilson and Houart, 2004) are therefore presumably not effective in quenching the effects of ectopic Wnt8.

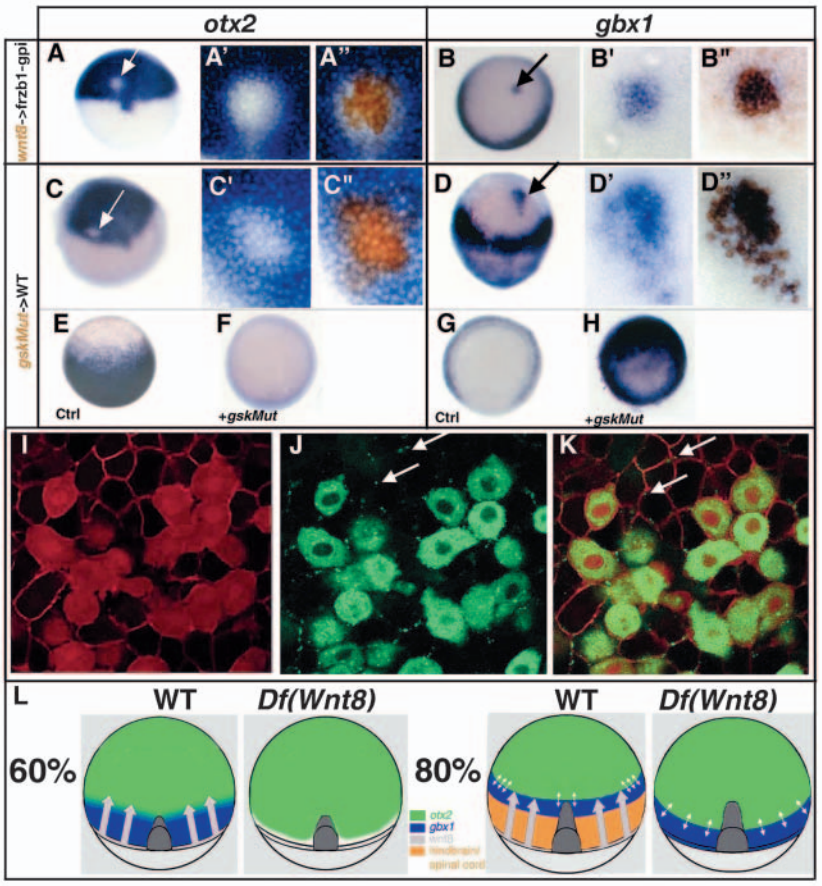



**Fig. 7.** Wnt8 represses *otx2* and induces *gbx1* in a non-cell-autonomous manner. (A-B″) Embryos containing cells derived from injected embryos with a lineage tracer (brown) and *wnt8* RNA (400 pg) into the animal pole of a host embryo injected with *Fzb1-gpi* RNA (200 pg). (C-H) A dominant-negative *gsk-3* can activate the Wnt pathway in a cell-autonomous manner. Embryos containing cells derived from injected embryos with a lineage tracer (brown) and *Xgsk-3K→R (gskMut)* RNA (400 pg). (A-A″,C-C″) Embryos stained for *otx2* expression. Close-up of the transplanted cells (A′-C′) before biotin staining and (A″-C″) after biotin staining. *otx2* repression occurs in the transplanted cells only and not in the surrounding host tissue. (B-B″,D-D″) Embryos stained for *gbx1* expression. Close-up of the transplanted cells (B′-D′) before biotin staining and (B″-D″) after biotin staining. *gbx1* is induced in the transplanted cells only. (E-H) Animal pole views. (E) Control embryos at 60% stained for *otx2*. (F) *gskMut*-injected embryos do not express *otx2*. (G) Control embryos at 60% stained for *gbx1*. (H) *gskMut*-injected embryos express *gbx1* ectopically mimicking ectopic *wnt* expression. (I-K) Embryos containing cells derived from injected embryos with a lineage tracer (red in the whole cell in I) and *wnt8-gfp* mRNA (green in J) (800 pg). The host embryos injected with palmitoylated m*RFP* mRNA (100 pg), which labels the cell membrane in red (I). (K) Overlay of (I) and (J). White arrows indicate the Wnt8 protein that has been secreted from the donor cells. (L) Wnt8 graded expression inhibits *otx2* and induces *gbx1* expression in the most posterior region of the embryos at 60% of epiboly. At this stage, the *otx2* and *gbx1* expression domains overlap slightly. Loss of Wnt8 leads to the loss of *gbx1* expression and to a posterior shift of the *otx2* expression domain. At 80% of epiboly, the *otx2* and *gbx1* expression domains are sharp and complementary, probably owing to mutual repressive interactions. In absence of Wnt8, the *gbx1* expression domain is established with a posterior shift. Its expression is complementary to the *otx2* expression domain.

Third, we tested if Wnt8 protein is found around expressing clones at an appreciable distance. We visualized Wnt8 protein around a transplanted clone by tagging it with GFP. A C-terminal Wnt8-GFP fusion protein is biologically active, as judged by its ability to induce *gbx1* and repress *otx2* following global injection (data not shown). We transplanted clones of *wnt8-gfp* overexpressing cells at pre-gastrula stages into wild-type hosts that were previously injected at the one-cell stage with a palmitoylated *RFP* mRNA (Iioka et al., 2004) to label all cell membranes (Fig. 7I), and analyzed Wnt8 localization by confocal microscopy. We observed GFP-fluorescent puncta in host cells around the transplanted cells, representing accumulated Wnt8-GFP protein (Fig. 7J, white arrows), mostly associated with the cell surface or extracellular matrix (Fig. 7K). Members of the Wnt family, including Wingless, are thought to tightly associate with membranes and heparan sulfate proteoglycan (Nusse, 2003). We can visualize accumulated Wnt8-GFP at a distance of about two cell diameters away from the donor cell clones, which is generally consistent with the genetically determined non-autonomous characteristics of Wnt8 (see Discussion). Because we observe *otx2* inhibition two to five cell diameters away from transplanted cell clones expressing Wnt8, it is likely that levels of Wnt8 protein below the detection threshold act at distances further away.

Taken together, our findings support a model where Wnt8 regulates, in a concentration-dependent manner, the location of the *otx2/gbx1* interface and hence, the position of the MHB organizer, via direct action of secreted Wnt8 emanating from the endomesodermal primordium at the blastoderm margin (Fig. 7L).

## Discussion

### Positioning of the MHB organizer is a direct consequence of posteriorization

In several vertebrates, including zebrafish, the position of the future MHB in the neuroectoderm is marked by the interface between cells expressing the Otx and Gbx transcription factors (Wassarman et al., 1997; Broccoli et al., 1999; Millet et al., 1999; Rhinn et al., 2003), raising the issue of how this interface is set up in turn. Here, we reported that AP positioning of the two expression domains of *gbx1* and *otx2* depends on

signals from lateral mesendodermal precursors, but not from axial mesendoderm. Explant cultures in chick previously suggested an involvement of axial mesendoderm in positioning MHB marker gene expression (Ang and Rossant, 1993; Shamim et al., 1999). In our studies, abolishing the formation of axial mesendoderm either in mutants or by ablation of its primordium, the shield, revealed however that the axial mesendoderm is not involved (Fig. 1). By contrast, lateral blastoderm margin transplants reveal that the marginal cells can induce *gbx1* and suppresses *otx2* in the surrounding tissue. This is in agreement with previous work, demonstrating that cells from the lateral margin can stimulate hindbrain gene expression (Woo and Fraser, 1997; Koshida et al., 1998; Sagerstrom et al., 1996; Momoi et al., 2003). Our work extends these findings to the earliest stages of neuroectoderm subdivision and positioning of the MHB organizer, and suggests that these events are directly linked to overall posteriorization of the neural primordium by signal(s) from the blastoderm margin.

The wealth of inductive interactions in early gastrulation stages makes it difficult to dissect the contribution of the various signaling pathways involved in posteriorization of the neural plate. This is clearly evident also for the formation of the *gbx1* and *otx2* expression domains in our loss-of-function experiments of the Nodal, Fgf or Wnt signaling pathways, all of which are active in the early blastoderm margin (Fig. 2). Specific interference with signal reception for individual signaling pathways in combination with a transplantation assay allowed us, however, to distinguish their contribution. Examination of cell clones that are 'blind' to any of the three signals enabled us to address whether such cells can respond with correct *otx2/gbx1* expression to positioning cues (Fig. 3). We find that Wnt signaling is crucially involved in setting up the *otx2* and *gbx1* expression domains, because cells that are 'blind' to Wnt signaling do not respond to AP positional information, causing *otx2* to be ectopically expressed in more posterior domains of the embryo. This is not the case when Nodal or Fgf signal reception is blocked, arguing that these signals have a different role. A role for Wnt molecules in repressing the *otx2* domain has been suggested previously in different experimental systems. Caudal chick neural plate cells revert to a rostral forebrain character when grown in vitro in the absence of a Wnt signal (Nordstrom et al., 2002). In *Xenopus*, treatment of animal caps with XWnt8 leads to a progressive posteriorization and a repression of anterior markers, including *otx2* (Kiecker and Niehrs, 2001). By contrast, Fgf and Nodal signaling seem to act in a more global context, because individual cells that cannot receive Fgf or Nodal signals respond correctly to AP positional signals. We suggest that Fgf and Nodal signaling are more indirectly involved in generating the *otx2/gbx1* interface: Nodal proteins, through their role in mesoderm formation (reviewed by Schier, 2001); and Fgfs, through their involvement in the induction of neural fate of vegetal ectoderm (Kudoh et al., 2004; Rentzsch et al., 2004) and in ventralization of the neuroectoderm (Fürthauer et al., 2004).

### The posteriorizing molecule Wnt8 mediates positioning of the MHB

Both our loss- and gain-of-function studies show that Wnt8, normally expressed in the blastoderm margin, is involved in the onset and correct positioning of the *gbx1* expression domain, and for the establishment of the posterior border of the *otx2* expression domain (Figs 4-6). Although we have focused here on *gbx1* and *otx2* as the most critical components for MHB development, it is likely that other target genes with posterior-specific expression would respond similarly to Wnt8, e.g. the *Cdx* genes. Our findings raise the issue of whether Wnt8 is directly involved in positioning the MHB primordium. We addressed this issue by injecting increasing amounts of *wnt8* mRNA, and by employing *wnt8*-overexpressing clones, which we find can repress *otx2*. Our clonal analysis in a Fzb1-gpi background, the cell-autonomous activation of the Wnt pathway by *Xgsk-3K→R* clonal analysis and our visualization of Wnt8-GFP all support the argument that Wnt8 regulates *otx2* and *gbx1* directly in a non-cell-autonomous manner (Fig. 7).

As in other vertebrates, mutually repressive interactions are thought to exist in zebrafish between *Otx* and *Gbx* genes (Rhinn and Brand, 2001; Rhinn et al., 2003) (M.R. and M.B., unpublished). Importantly, several findings argue that feedback regulation is preceded by a phase of direct regulation of both *otx2* and *gbx1* via Wnt8. First, we found that Wnt8 can regulate both genes independently, because it can induce *gbx1* and regulate *otx2* prior to the establishment of the feedback loop between them around 70% epiboly. In the Df(*wnt8*) mutants and in *wnt8* morphants, posterior expansion of *otx2* is evident prior to the 70% stage when these mutually repressive interactions become evident. Second, *wnt8*-overexpressing clones can repress *otx2* without inducing *gbx1,* and Wnt 'blind' cells ectopically express *otx2*. Analysis of *wnt8* showed that the gene is crucially involved in the patterning of mesoderm and neural ectoderm (Christian et al., 1991; Lekven et al., 2001; Erter et al., 2001). *gbx1* activation coincides with the involution of the forming mesendoderm (Rhinn et al., 2003), raising the possibility that vertical signaling from the involuted mesendoderm to the overlying ectoderm could also be involved in this process. Our results argue against this possibility. First, in MZ*oep* embryos, where no dorsolateral mesendoderm involution occurs, *gbx1* is induced and *otx2* expansion does not extend further posteriorly, towards the lateral margin. Second, *gbx1* expression throughout the complete epiblast is observed when *wnt8* is expressed ectopically with a limited upregulation of mesendodermal markers (Kelly et al., 1995) (Fig. 6M,N). This suggests that the whole embryo can respond to *wnt8* signaling to induce *gbx1* in the absence of mesendoderm. Third, we found that in transplantations of *wnt8*-expressing cells, *gbx1* is induced and *otx2* is repressed without new mesoderm induction, consistent with previous findings that Wnt signaling can induce posterior neural markers in the absence of mesendoderm (McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001). Altogether these findings suggest that *wnt8* plays a key role in the activation of *gbx1* and repression of *otx2*, independent of its role in mesoderm patterning.

### Graded activity of Wnt8 signaling in the early neural plate

How does Wnt8 participate in positioning of the MHB organizer? *wnt8* is expressed in the marginal cells and hypoblast and two receptors, *fz8c* and *fz9*, are detected in both hypoblast and epiblast (Momoi et al., 2003). Conceivably, Wnt8 is transmitted in a planar fashion through the

neuroectoderm. This idea is supported by the clonal analysis of *wnt8* overexpressing cells: *gbx1* is activated in the host tissue one or two cells distant from the transplanted cells, and *otx2* is repressed four or five cells distant from the transplanted cells (Fig. 6). In unmanipulated neuroectoderm, the onset of *gbx1* expression occurs close to the *wnt8* domain with little or no overlap, and the *otx2* expression domain is situated eight to ten cell diameters away from the *wnt8* domain at 60% of epiboly. Thus, the *wnt8* expression domain is appropriately located to generate a graded morphogenetic Wnt8 signal that regulates the expression of *gbx1* and *otx2* genes in vivo. This finding is more generally consistent with the ability of Wnt molecules to form gradients and to activate target genes in a concentration-dependent manner, as in the *Drosophila* wing imaginal disc, where expression of *wingless* target genes like *neuralized*, *distalless* and *vestigial* depends on the distance from wingless-expressing cells (Zecca et al., 1996; Strigini and Cohen, 2000). Similarly, in the unmanipulated zebrafish neuroectoderm, the *otx2* and the *gbx1* domains are located at different distances from the Wnt8 source at the lateral blastoderm margin. Following global misexpression experiments, different Wnt8 doses can differentially regulate *otx2* and *gbx1* expression: *wnt8* ectopic expression can induce *gbx1* expression at low/intermediate doses, but represses at high doses. Conversely, *otx2* is increasingly repressed with increasing *wnt8* concentration. Similarly, around *wnt8*-expressing clones, *gbx1* is induced at a distance of one or two cells around the clone, whereas *otx2* is repressed at a distance of four or five cells. This suggests that a lower Wnt8 concentration is needed to repress *otx2* than to induce *gbx1*. Altogether, these observations suggest that Wnt8 has properties of a morphogen whose activity is required to correctly position the *otx2/gbx1* interface, and probably other target genes in the forming neural plate. Our observation of secreted Wnt8-GFP protein emanating from clones of producing cells is generally consistent with this possibility. Distribution of another signaling molecule in the early neural plate, Fgf8, is carefully controlled by endocytosis (Scholpp and Brand, 2004). It will be interesting to determine if Wnt8 protein is indeed distributed in a graded fashion, and which mechanisms control this distribution. In mice, *Wnt8* is expressed in the posterior epiblast of early primitive streak-stage embryos (Bouillet et al., 1996); although its function is unknown, Wnt8 may therefore serve a similar function as proposed here.

Other studies also suggest that a Wnt/β-catenin signaling gradient may underlie AP patterning in the neuroectoerm. In *Xenopus* gastrula, Wnt activity declines in the neural plate from high caudal to low rostral levels (Kiecker and Niehrs, 2001). In favor of graded Wnt activity are also observations in chicken where neural plate explants express different regional markers in response to different concentrations of Wnt-conditioned medium (Nordstrom et al., 2002). In zebrafish, results by Momoi and collaborators (Momoi et al., 2003) did not detect graded Wnt activity. These authors observed at 70% of epiboly stage a nuclear localization of β-catenin along the AP axis, but only in *wnt8*-expressing cells in the blastoderm margin. However, given the timing of *gbx1/otx2* onset and the genetic requirement for Wnt8 documented here, we predict that the Wnt gradient is generated in the neural plate already prior to 70% of epiboly. Conceivably, a low level of β-catenin nuclear translocation might suffice to transmit the Wnt8 signal that was not detectable in the assay by Momoi et al. (Momoi et al., 2003). In summary, our data argue that Wnt8 acts without a relay mechanism in the regulation of *otx2* and *gbx1*, and we hence strongly favor the idea that during the early and mid-gastrulation stages in zebrafish, similar to *Xenopus* and *Drosophila*, there is a graded Wnt8 signal that is generated from the blastoderm margin (see also Dorsky et al., 2003). The recent description of Tcf4-binding sites in the enhancers driving *Otx2* expression in the mouse anterior neural plate suggests that one level of this regulation occurs at the level of the *Otx2* promoter (Kurokawa et al., 2004).

The suggested Wnt8 action from the lateral blastoderm margin, located at the posterior edge of the forming neural plate, may be antagonized from the anterior side forming at the animal pole, through inhibition that is required for assigning cell fate in the forebrain neural plate (Kim et al., 2000; Onai et al., 2004; Wilson and Houart, 2004). One possibility is that these interactions start quite early when the distances between cells of the future anterior and posterior neural plate are still small. The Wnt8 signal is then integrated to process neural AP patterning and positioning of the MHB primordium at 60% of epiboly (Fig. 7L). Positioning of the future MHB organizer is thus achieved by Wnt8 setting up the complementary *gbx1* and *otx2* expression domains before mutual interaction between them sharpens the interface, around 70-80% of epiboly (Rhinn et al., 2003), and before the establishment of the complex regulatory cascade conferring to the MHB cells their organizing capacity (Fig. 7L).

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### Supplementary material

Supplementary material for this article is available at
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