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Specification of an anterior neuroectoderm patterning by Frizzled8a-mediated Wnt8b signalling during late gastrulation in zebrafish

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SUMMARY

Wnts have been shown to provide a posteriorizing signal that has to be repressed in the anterior neuroectoderm for normal anteroposterior (AP) patterning. We have previously identified a zebrafish *frizzled8a* (*fz8a*) gene expressed in the presumptive anterior neuroectoderm as well as prechordal plate at the late gastrula stage. We have investigated the role of Fz8a-mediated Wnt8b signalling in anterior brain patterning in zebrafish. We show that in zebrafish embryos: (1) Wnt signalling has at least two different stage-specific posteriorizing activities in the anterior neuroectoderm, one before mid-gastrulation and the other at late gastrulation; (2) Fz8a plays an important role in mediating anterior brain patterning; (3) Wnt8b and Fz8a can functionally interact to transmit posteriorizing signals that determine the fate of the posterior diencephalon and midbrain in late gastrula embryos; and (4) Wnt8b can suppress *fz8a* expression in the anterior neuroectoderm and potentially affect the level and/or range of Wnt signalling. In conclusion, we suggest that a gradient of Fz8a-mediated Wnt8b signalling may play crucial role in patterning the posterior diencephalon and midbrain regions in the late gastrula.

Key words: Fz8a, Wnt8b, Anterior neuroectoderm, Brain patterning, Eye primordia, Diencephalon, Midbrain, Zebrafish

INTRODUCTION

In vertebrates, several models have been proposed to explain anteroposterior (AP) patterning of the nervous system. One of these, which has attracted considerable attention, is the 'activation/transformation' model proposed by Nieuwkoop (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954; Saxen, 1989; Sasai and De Robertis, 1997; Sive et al., 1989), based on experiments conducted in amphibian embryos. The model proposes that the initial signals of neural induction ('activation') also simultaneously define a forebrain state at the early gastrula stage, and later, a 'transforming' signal from the chordamesoderm converts some of this initially specified anterior neuroectoderm to more posterior fates (reviewed by Saxen, 1989).

Also in amphibians, β-catenin-mediated Wnt signalling has been shown to be involved in body axial patterning during two distinct developmental stages: one before and one after the midblastrula transition (MBT) (reviewed by Niehrs, 1999). Wnt signalling before MBT is required for the initiation of dorsal axis formation (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991), whereas a later phase of Wnt signalling after MBT appears to be involved in anteroposterior patterning of the neural axis (Christian and Moon, 1993; McGrew et al., 1995). Ectopic expression of *Xenopus* Wnts (Saint-Jeannet et al., 1997; Fredieu et al., 1997; McGrew et al., 1995; Chang and Hemmati-Brivanlou, 1998; Popperl et al., 1997), β-catenin (McGrew et al., 1995) or the artificial activation of the Wnt pathway by LiCl treatment in amphibian embryos (Fredieu et al., 1997) after MBT all interfere with head development. These observations suggest a negative function of Wnt signalling for anterior neural fate determination. The identification of Wnt inhibitors (Cerberus, Frzb1 and Dickkopf 1) (Bouwmeester et al., 1996; Leyns et al., 1997; Wang et al., 1997; Glinka et al., 1998) as head inducers also suggests that blocking Wnt signalling in the anterior neuroectoderm is a prerequisite for patterning the brain along the AP axis. More recently, it has been suggested that repressor activity of Tcf3 encoded by the *headless* (*hdl*) locus, which binds to Wnt target genes, is an essential factor for head formation in zebrafish (Kim et al., 2000). These observations support the proposal of the 'two-inhibitor model': that repression of Wnt signalling is required for vertebrate head induction (Glinka et al., 1997).

Despite the prevailing idea that anterior neuroectodermal fate is determined prior to posterior neuroectodermal identity, it is not known what genes help to subdivide the anterior neuroectodermal field into various brain regions, including

telencephalon, diencephalon, and eye primordium, midbrain and mid-hindbrain boundary (MHB).

Previously, we have isolated a cDNA clone for zebrafish *fz8a* that is expressed in the neuroectoderm as well as in the organiser (Kim et al., 1998). Zebrafish *fz8a* is expressed in the entire neuroectoderm at early gastrula and by late gastrula, its expression domain is gradually reduced until it becomes restricted to the regions of the anterior neuroectoderm fated to contribute to the telencephalon, diencephalon and eye primordium (Woo and Fraser, 1995). Similar to Fz8a expression in the anterior neuroectoderm, Wnt1 and Wnt8b, members of Class I Wnt family (which signal via β-catenin), also start to be expressed at the presumptive midbrain, including MHB in the late gastrula stage of zebrafish embryos (Kelly and Moon, 1995; Kelly et al., 1995). These observations raise the possibility that a gradient of Wnt signalling established within the anterior neurectoderm by late gastrulation plays contributes to patterning in the neurectoderm. To explore this possibility, we have studied the roles of Fz8a-mediated Wnt signalling in fate determination of the anterior neuroectoderm.

In this report, we show that the rostral and caudal parts of the zebrafish anterior brain are negatively and positively regulated, respectively, by Wnt signals through the Fz8a receptor. We also suggest that different thresholds of Fz8a-mediated Wnt8b signalling in the anterior neuroectoderm during late gastrulation is crucial for the proper patterning of the posterior diencephalon and midbrain

MATERIALS AND METHODS

Fish maintenance and sampling

Zebrafish were raised, maintained and staged as described in *The Zebrafish Book* (Westerfield, 1995). Embryos were obtained by spontaneous spawning and appropriate stages of the embryos were fixed with 4% paraformaldehyde in PBS and dechorionated with watchmakers' forceps.

Plasmid constructions and RNA injection

The zebrafish *fz8a*-coding region (Kim et al., 1998) was subcloned into the *Bam*HI and *Xho*I site of plasmid pCS2(+) to generate plasmid pCS2(+)-Fz8a. To construct a dominant negative form of Fz8a (Fz8a-CRDTM), pCS2(+)-Fz8a plasmid DNA was digested with *Dra*I/*Stu*I to remove the DNA sequences encoding transmembrane domains (second to seventh) and intracellular domain of Fz8a. The resulting larger DNA fragment was self-ligated to generate Fz8a-CRDTM that encodes the first 267 amino acids including the CRD domain with the first transmembrane domain. To construct a secreted dominant negative form of Fz8a (Fz8a-CRD), DNA sequence encoding seven transmembrane domains and an intracellular domain of Fz8a was removed from pCS2(+)-Fz8a plasmid DNA by digestion with *Stu*I/*Bss*HII. To construct a Fz8a-CRD- EGFP, the resulting Fz8a-CRD that encodes the first 198 amino acids including the CRD domain was fused with EGFP cDNA (Clontech) and then placed at the downstream of zebrafish hsp70-4 heat-inducible promoter (Lele et al., 1997). A form of Tcf3 (dNTCF3), pCS3+MT-ΔTCF3, that lacks the first 47 amino acids of β-catenin-binding domain of Tcf3 (Kim et al., 2000), was used to repress Wnt target genes. Capped mRNAs for injections were synthesised from linearised plasmids pCS2(+)-Fz8a, *fz8a-CRDTM*, pT7Ts-Wnt8b (a gift from R. T. Moon) and pCS3+MT-ΔTCF3 using an in vitro transcription kit with $m^7G(5')ppp(5')G$ (Boehringer Mannheim). Capped mRNAs were diluted in 0.1 M KCl solution containing 0.5% Phenol Red, 0.5-5 pg of *wnt8b*, 20-300 pg of *fz8a-CRDTM*, 5-350 pg of *fz8a* and 12-50 pg of *dNTCF3* were injected into one-cell stage embryos. Morpholino oligonucleotides for *fz*8a (*fz8a*-MO, 5′-AGTTCAGTTCAGATGTTGCAGAG-3′) and *wnt8b* (*wnt8b*-MO, 5′-ACTTTTCTTCACCTTTCAC-3′), and four base pair mismatched control morpholinos for *fz8a* (*4misfz8a*-MO, 5′-AGTTGAGTTCTGATGATGCACAG-3′) and for *wnt8b* (*4miswnt8b*-MO, 5′-ACTTATCTACACGTTTCTC-3′), which are designed to have no predicted internal hairpins and avoiding the presence of four consecutive G nucleotides, were purchased from Gene-Tools, LLC (Corvallis, OR). *fz8a*-MO (10 ng/embryo) and *wnt8b*-MO (10 ng/embryo) were solubilised in 0.1 M KCl solution prior to use and then injected into each of the one-cell stage zebrafish embryos. Mismatched bases are underlined.

Expression of secreted dominant negative form of Fz8a under the heat-inducible promoter

For stage-specific expression of a secreted dominant negative form of Fz8a at late gastrula stage, Fz8a-CRD-EGFP DNA (50 pg/embryo) was injected into one-cell stage embryos and cultured at 26°C. Embryos were cultured to 75% epiboly stage, subjected to heat-shock at 37°C for 1 hour and then further incubated at 28.5°C. Embryos that showed higher level of EGFP expression were selected under the fluorescence microscope, further cultured until seven-somite stage, fixed and subjected to in situ hybridisation.

Lithium chloride treatment

To collect the same staged-embryos, embryos were harvested within 10 minutes after mating. For LiCl treatment, various stages of embryos (0.3 g sea salt, 2 mg Methylene Blue in 1 l water) were exposed to 0.3 M LiCl in egg water for 15-20 minutes at 28°C, washed with egg water and then allowed to develop at 28.5°C.

Whole-mount in situ hybridisation

Antisense digoxigenin-labelled RNA probes for *anf*, *eng2*, *fkd5*, *fz8a*, *opl*, *pax2*, *pax6*, *rx1*, *rx3*, *six3* and *wnt8b* were produced using a DIG-RNA labelling kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridisation and detection with an anti-digoxigenin antibody coupled to alkaline phosphatase (Vector Laboratories) were performed as described by Jowett and Lettice (Jowett and Lettice, 1994). Double in situ hybridisation was performed as described by Hauptmann and Gerster (Hauptmann and Gerster, 1994). Embryos were photographed using a Nikon coolpix900 digital camera system attached to a Zeiss Axioskop microscope.

Transplantation

For transplantation, one-cell stage donor embryos were injected with 1 nl of RNA solution [4 ng/μl, *wnt8b* RNA; 5 μg/μl, lysinated tetramethylrhodamine-dextran (M_r 70,000, Molecular probes); 4 μg/μl, lysinated biotin-dextran (M_r 2,000,000, Molecular Probes)] and further cultured to the blastula stage. Transplantation of blastomeres at midblastula stage was performed into same-staged sibling hosts. A small group of 5-10 cells from a labelled donor embryo was gently drawn up into the transplantation pipette and transplanted into the blastoderm of the host embryos. The host embryos were allowed to develop at 28.5°C in Ringer's solution. Host embryos at 100% epiboly stage were fixed in 4% paraformaldehyde and whole-mount in situ hybridisation was performed as described above. In order to detect donor-derived cells in the host after in situ hybridisation, the host embryos were re-fixed, and then processed for biotin detection using avidin and biotinylated peroxidase complex method (Vectastain ABC kit).

RESULTS

Overlapped expression of *fz8a* and *wnt8b* genes in the anterior neuroectoderm of late gastrula embryos

In the anterior neuroectoderm of the 90% epiboly embryos, low

levels of *wnt8b* transcripts are first detected at the putative midbrain region. The anterior boundary of the *wnt8b* expression domain initially overlaps with the posterior boundary of the *fz8a* expression domain, thus *fz8a* and *wnt8b* mRNA transcripts were commonly detected in the anterior region of putative midbrain territory at 90% epiboly (Fig. 1A,C). By the end of gastrulation (100% epiboly), however, *fz8a* expression gradually decreases at its posterior boundary, where its expression initially overlapped with *wnt8b*, and eventually the two adjacent expression domains of *fz8a* and *wnt8b* are separated by a gap (Fig. 1B,D). However, subsets of cells expressing *fz8a* are still detected in the anterior region of *wnt8b* expression domain (Fig. 1C,D). The proximity of *fz8a* and *wnt8b* expression domains in the future anterior brain region suggests a potential role for Fz8a-mediated Wnt8b signalling in anterior brain patterning. The *fz8a*-expressing domain was found to encompass the expression domains for several regional anterior neuroectoderm markers: *opl* for telencephalon (Grinblat et al., 1998) (Fig. 2A), *anf* (Kazanskaya et al., 1997) for telencephalon and diencephalon (Fig. 2B) and *six3* (Kobayashi et al., 1998) for telencephalon, retina and the anterior tip of the prechordal plate (Fig. 2C). This suggested a specific role for Fz8a and Wnt8b in forebrain and midbrain patterning.

LiCl treatment at gastrula stage mimics the *wnt8b* misexpression

Lithium chloride (LiCl) treatment of embryos after MBT can stimulate the β-catenin-mediated class I Wnt signalling pathway (Klein and Melton, 1996; Hedgepeth et al., 1997) and mimic the loss of brain structures caused by overexpression of X*wnt*8 (Fredieu et al., 1997). Therefore, we further examined whether *wnt8b* overexpression and LiCl treatment exert similar effects on anterior brain development. One-cell stage embryos were injected with a low dose of *wnt8b* mRNA (2-3 pg/embryo) and allowed to grow until 100% epiboly. Concurrently, uninjected shield stage embryos were exposed to LiCl, cultured until the completion of epiboly (9.5 hpf), at which the effects of *wnt8b* overexpression and LiCl treatment on the expression of anterior neuroectodermal markers (*opl*, *anf*, *six3*, *fz8a*) were analysed. A complete loss of all anterior neuroectoderm markers was seen in the anterior neuroectoderm of both *wnt8b*-injected (Fig. 2F-I) and LiCl-treated embryos (Fig. 2K-N). However, expression of *six3* and *fz8a* in the anterior tip of the prechordal plate was not severely affected by either treatment (Fig. 2H,I,M,N). Nevertheless, both LiCl-treated and *wnt8b*-injected embryos at 25 hpf showed very similar defects in the anterior brain structure (Fig. 2J,O), suggesting that LiCl treatment at the onset of gastrulation induces defects in forebrain patterning similar to that caused by overexpression of *wnt8b*.

Stage-specific effect of ectopic Wnt signalling on neuroectoderm patterning

To investigate the potential role of Wnt signalling in patterning the anterior brain during late gastrulation, zebrafish embryos were exposed to LiCl at the various gastrula stages. When embryos were exposed to LiCl at stages earlier than mid-gastrula (8 hpf), a progressive loss of neural structures from rostral to caudal was observed; most embryos lost the forebrain and eye primordia (Fig. 3B,C). By contrast, LiCl treatment at later gastrula stages had a different effect and resulted in embryos with smaller eyes (Fig. 3D,E). Finally, embryos treated with LiCl at the 100% epiboly appeared almost normal in their eye and forebrain structure (Fig. 3F). These results suggest that Wnt signalling has two different stage-specific posteriorizing activities, one before mid-gastrulation and the other at late gastrulation: activation of Wnt activity in early gastrula can transform the whole forebrain territory into midbrain. By contrast, increased Wnt activity after late gastrula stage has limited posteriorizing activity, which can transform rostral fate into more caudal fate within anterior brain region, as indicated by the caudal defect of eye primordia as well as accompanying posterior diencephalon expansion (see Fig. 3M-X′). These stage-dependent effects of LiCl are analysed statistically in Table 1.

Table 1. Degree of loss of eye by serial LiCl treatment during gastrulation in zebrafish

	Phenotype (*n*)		
	Absent eye	Small eye	Normal eye
Dome	30	0	0
Shield	29	1	0
60% epiboly	28	2	0
70% epiboly	16	13	1
80% epiboly	11	15	4
90% epiboly	2	22	6
95% epiboly	0	18	12
100% epiboly	0	8	20
Tail bud	0	0	29

Embryos are exposed to 0.3 M LiCl at the indicated stages for 15 minutes at 26°C. They were then cultured to nine-somite stage and scored for loss of eye structure with microscopic observation. Small eye is illustrated in Fig. 3D,E,J,K.

To confirm the defect of anterior structures caused by LiCl, the changes in *rx1* (Chuang et al., 1999) and *eng2* (Fjose et al., 1992) expression in eye primordia and midbrain, respectively, were further examined. In embryos exposed to LiCl at the early gastrula stage (shield stage), *rx1* expression in the eye primordia was completely abolished, but the midbrain region marked by *eng2* was remarkably expanded (Fig. 3H). By contrast, embryos treated with LiCl at the late gastrula stage (80% and 90% epiboly) showed small eyes with decreased *rx1* expression, without any notable increase of *eng2* expression in the midbrain (Fig. 3J,K). Finally, LiCl treatment at the end of gastrula stage (100% epiboly) did not induce any significant change of *rx1* and *eng2* expression, suggesting that activation of Wnt signalling at this stage has little effect on the size of the eye and midbrain (Fig. 3L). These results further support our observation that activation of Wnt signalling has stage-specific effects during gastrulation.

Posteriorizing activity of Wnt signalling at late gastrula regulates anterior brain patterning

To further characterise the stage specific effects of Wnt signalling on the patterning in the anterior neuroectoderm, we analysed the changes of *pax6* and *pax2* expression in embryos treated with LiCl; *pax6* and *pax2* have been reported to regulate forebrain and midbrain patterning (Krauss et al., 1991; Matsunaga et al., 2000; Schwarz et al., 1999; Urbanek et al., 1997; Warren and Price, 1997). At the one-somite stage, *pax6* is expressed in a graded pattern in the presumptive anterior

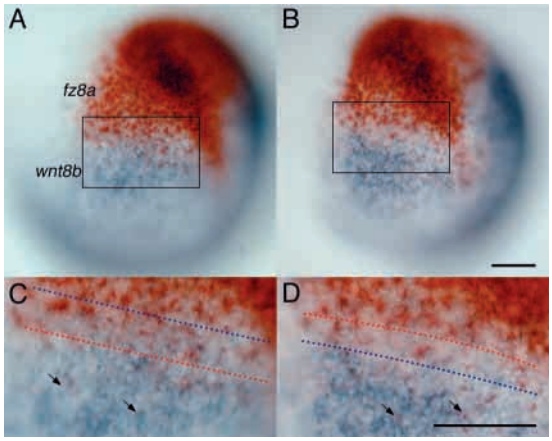

Fig. 1. The expressions of *fz8a* and *wnt8b* are overlapped during late gastrulation of zebrafish embryos. (A-D) Dorsolateral views of embryos that were double stained for *fz8a* (red) and *wnt8b* (blue) mRNA. (A) At 90% epiboly stage, *wnt8b* started its expression in the posterior boundary of *fz8a* expression domain. (B) At 100% epiboly stage, the posterior boundary of *fz8a* expression domain, where *wnt8b* expression was overlapped, was gradually decreased; thus, the two adjacent expression domains for *fz8a* and *wnt8b* were separated. (C,D) Magnified images of insets from A and B, respectively. The broken red and blue lines indicate the posterior boundary of *fz8a* expression and anterior boundary of *wnt8b* expression, respectively. Arrows indicate *fz8a*-expressing cells remained in the *wnt8b* expression domain. Scale bars: in A, 100 μm for A,B; in D, 100 μm for C,D.

brain; it is highly expressed in the putative posterior diencephalon (arrowhead in Fig. 3M) and weakly expressed in the more rostral brain including the telencephalon, eye primordia and anterior diencephalon. This graded expression pattern of *pax6* in the posterior diencephalon is also maintained at the seven-somite stage (Fig. 3S,S′). When embryos were exposed to LiCl at the early gastrula stage (shield stage), the putative MHB domain (marked by *pax2* expression) was markedly expanded anteriorly, while rostral brain structures (marked by *pax6*) were completely lost in one-somite stage embryos (Fig. 3N). A similar expansion of the *pax2* and loss of *pax6* expression domains was seen at the seven-somite stage (Fig. 3T,T′). LiCl treatment at the 70% epiboly also caused the expansion of the *pax2* domain with reduction of the *pax6* domain (Fig. 3O,U,U′).

In contrast to the changes induced by LiCl treatment during early gastrulation, activation of Wnt signalling at the late gastrula stage (80 and 90% epiboly) induced a rostral expansion of the *pax6* expression without causing much of a change in the size of the MHB domain marked by *pax2* expression (Fig. 3P,Q). A similar change was seen at the seven-somite stage (Fig. 3V,W,V′,W′). However, *pax2* and *pax6* expressions in embryos treated with LiCl at the end of gastrulation (100% epiboly) were not changed (Fig. 3R,X,X′), thus almost comparable with those in untreated embryos (Fig. 3M,S,S′).

The anteriorly expanded *pax6* expression in the diencephalon suggests that Wnt signalling activated at the late gastrula stage increases in the relative size of posterior diencephalic character within the diencephalon territory. Because the length from the anterior tip to the MHB in the same developmental stage of embryos is constant, the changes in the relative ratios for sizes of each territory of the anterior brain domains should occur reciprocally. In our study, the expansion of posterior fates (posterior diencephalon) at late gastrula resulted in a concomitant reduction of the relative size of the anterior fates (ventral diencephalon and eye primordia) (Fig. 3V,W). These results, taken together, indicate that Wnt signalling in the late gastrula particularly contributes to anterior brain patterning by imposing a posteriorizing Wnt signal on the posterior region of anterior neuroectoderm.

Ectopic expression of *fz8a* reduces eye size

To investigate whether *fz8a* in the late gastrula contributes to anterior brain patterning by posteriorizing anterior neuroectoderm from caudal to rostral, we conducted gain-of-function and loss-of-function experiments for Fz8a (Fig. 4; Table 2). Injection of *fz8a* mRNA into the one-cell stage embryos reduced the sizes of the eye primordia and anterior head structures (87%, *n*=108; Fig. 4B,F) similar to the results seen in embryos exposed to LiCl at late gastrula

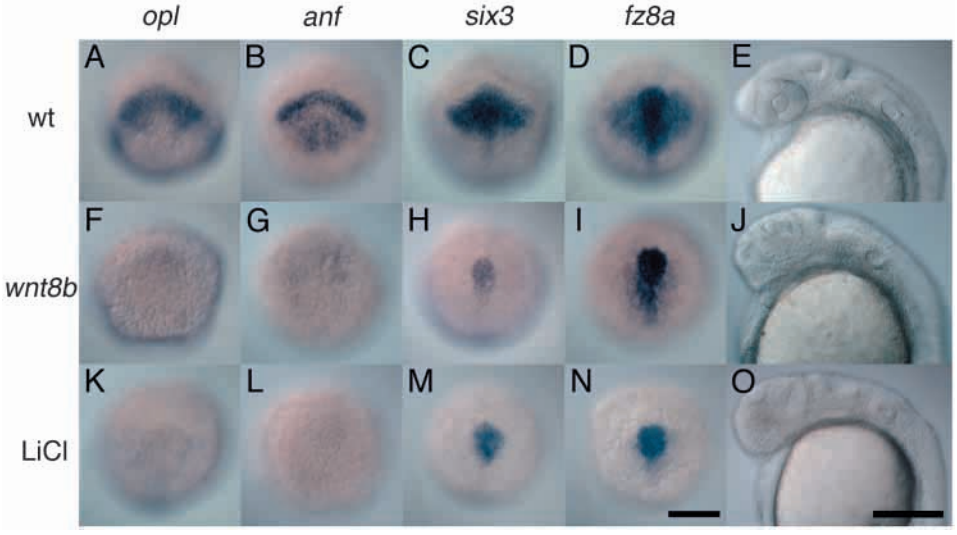

Fig. 2. LiCl treatment at gastrula mimics *wnt8b* overexpression. (A-D) Wild-type (control) embryos at 100% epiboly are stained with anterior neuroectoderm markers, *opl*, *anf*, *six3* and *fz8a*. (E) Control embryo at 25 hpf. (F-I) One-cell embryos were injected with low dose of *wnt8b* mRNA (2 pg/embryo), further cultured to 100% epiboly and then their expression patterns of the anterior neuroectoderm markers analysed. (J) 25 hpf embryo injected with *wnt8b*. (K-N) Embryos were exposed to LiCl at the shield stage, allowed to grow until 100% epiboly and then their expression patterns of the anterior neuroectoderm markers were analysed. (O) 25 hpf embryo treated with LiCl at shield stage. Scale bars: in N, 250 μm for A-D,F-I,K-N; in O, 250 μm for E,J,O.

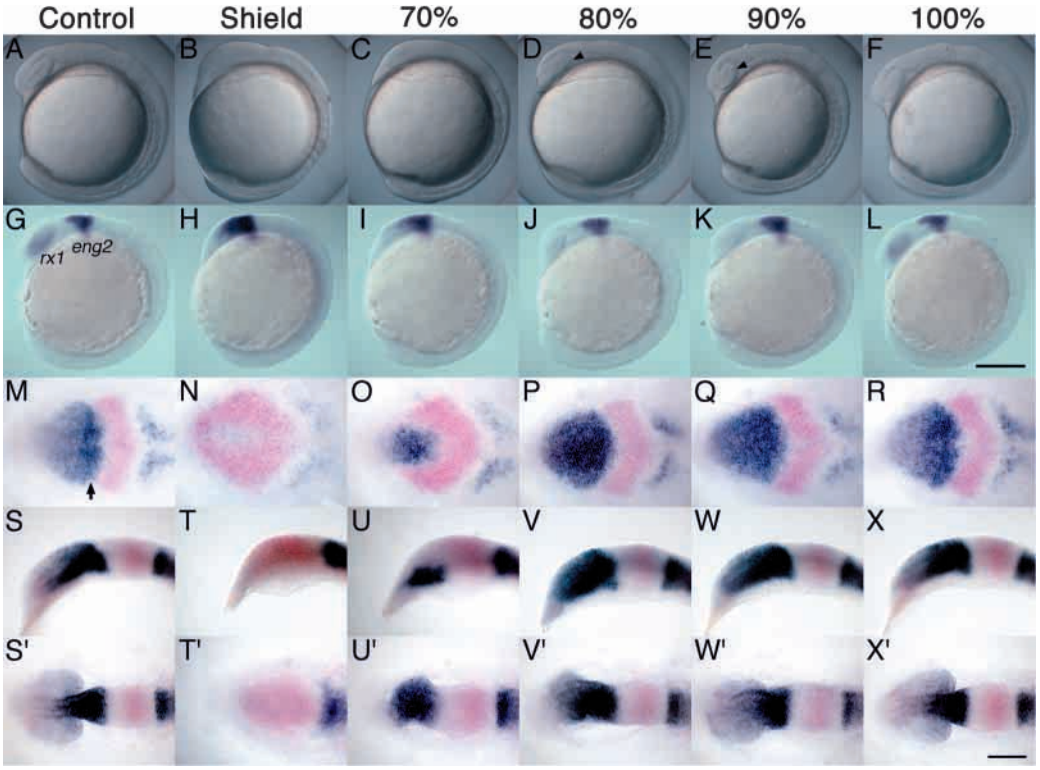

Fig. 3. Gain of Wnt signalling is stage specific. Various gastrula stage embryos were exposed to LiCl and the gain of Wnt activity was analysed at the nine-somite stage embryos (A-L). Untreated embryos (control) (A,G), embryos treated with LiCl at shield (B,H), 70% epiboly (C,I), 80% epiboly (D,J), 90% epiboly (E,K) and 100% epiboly stage (F,L). The expansion of midbrain and loss of eye primordia phenotypes are verified with *eng2* and *rx1* expressions in embryos exposed to LiCl during gastrulation (H-L) and in untreated control (G). Embryos exposed to LiCl before the mid-gastrula stage exhibit a complete loss of eye primordia (B,C,H,I), while embryos exposed to LiCl at late gastrula onwards show partial loss of eye primordia (D,E,J,K). By contrast, embryos exposed to LiCl at the end of gastrulation show almost normal anterior brain structures including eye primordia (F,L). Arrowheads indicate caudal defect in eye primordia. Scale bar: 250 μm. Anterior brain patterning is regulated by posteriorizing activity of Wnt signalling at late gastrula (M-X′). Changes of *pax6* expression (blue) in the diencephalon and *pax2* expression (red) in the MHB were analysed in the one-somite (M-R) and seven-somite stage embryos (S-X′), respectively. In diencephalon, *pax6* expression is most abundant in the posterior diencephalon domain (arrow) and gradually decreases anteriorly. Untreated embryos (control) (M,S,S′), embryos exposed to LiCl at 60% (N,T,T′), 70% (O,U,U′), 80% (P,V,V′), 90% (Q,W,W′) and 100% epiboly (R,X,X′). In embryos treated with LiCl at early gastrula, the forebrain (telencephalon, ventral diencephalon and posterior diencephalon) and eye primordia fates are lost or severely reduced, but MHB domain is expanded anteriorly (N,O,T,U,T′,U′). By contrast, embryos exposed to LiCl at 80% (P,V,V′) and 90% epiboly (Q,W,W′), the posterior diencephalon fate is expanded without increasing MHB domain. Embryos exposed to LiCl at the 100% epiboly show almost normal anterior brain patterning at the seven-somite stage (X,X′). Scale bar: 125 μm.

(Fig. 3D,E). In accordance with this, overexpression of the dominant-negative form of Fz8a (Fz8a-CRDTM, Fig. 4I) caused the expansion of eye primordia and anterior head structures (84.5%, *n*=116; Fig. 4C,G). These observations implicate Fz8a in contributing to Wnt signalling that is required for proper anterior brain patterning.

Recently, it was shown that *hdl*, a zebrafish homologue of *Tcf3*, is a repressor of Wnt target genes (Kim et al., 2000). To further demonstrate that repression of Wnt signals induces an expansion of anterior fates, a synthetic RNA encoding a form of *hdl* (dNTCF3) that lacks its β-catenin

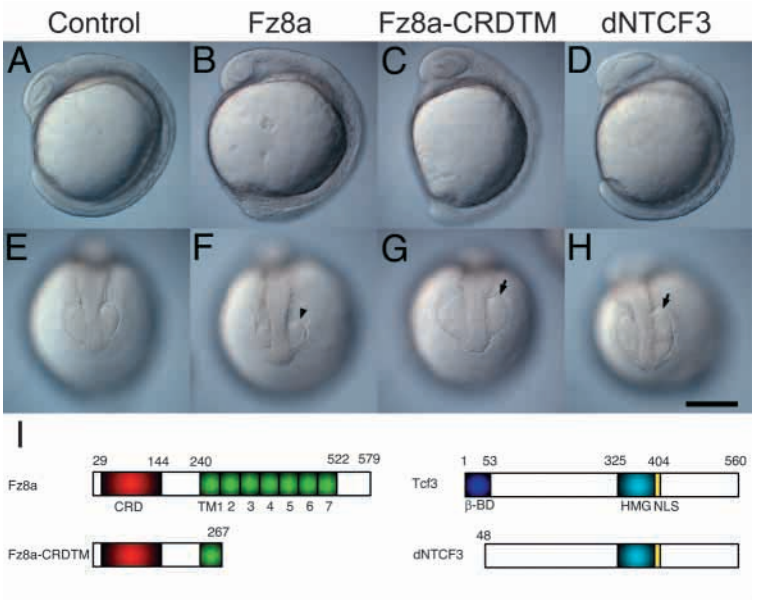

Fig. 4. Ectopic expression of *fz8a* reduces eye size, while Fz8a-CRDTM induces large eyes. Wild-type embryos (A,E) and embryos injected with *fz8a* (300 pg/embryo; B,F), *fz8a-CRDTM* (250 pg/embryo; C,G) and *dNTCF3* (25 pg/embryo; D,H) were allowed to grow until the nine-somite stage. Changes of the eye size are shown in lateral view (upper panel) and anterior view (lower panel). Arrowhead indicates caudally reduced eye primordia in *fz8a*-injected embryos (F), and arrows denote the enlarged eye primordia in embryos injected with *fz8a-CRDTM* (G) or *dNTCF3* (H). Scale bar: 250 μm. (I) Structure of cDNA constructs encoding dominant negative forms of mutant protein used for injection experiments. CRD, cysteine rich domain; TM, transmembrane domain; β-BD, β-catenin binding domain; NLS, nuclear localisation signal.

Table 2. Effects of *fz8a, Fz8a-CRDTM, dNTCF3* and *wnt8b* RNA injection

Sample	RNA injected (pg/embryo)	Eye phenotype (%)				Death by dorsalizaton*	Total (*n*)
		Absent	Small	Normal	Large		
Fz8a	350	0	87	3.7	0	9.3	108
Fz8a-CRDTM	300	0	0	3.4	84.5	12.1	116
dNTCF3	12	0	1.9	33.3	42.6	22.2†	54
Wnt8b	0.5	22.2	61.1	16.7	0	0	169
	2	90.6	7.5	1.9	0	0	106
	5	95.5	4.5	0	0	0	88
Wnt8b+Fz8a‡	0.5+5	8.5	18	45.5	0	28	200
	0.5+20	8.3	13.5	26.1	0	52.1	96
	2+20	0	2.1	17	0	80.9	94
Wnt8b+Fz8a-CRDTM§	2+20	27.9	65.1	7.0	0	0	86
	2+40	0	6.5	93.5	0	0	62
β-galactosidase	200	0	0	100	0	0	80

Synthetic mRNAs (*wnt8b*, *fz8a*, *Fz8a-CRDTM*, *dNTCF3* and β-galactosidase) were injected into the blastomere of one-cell stage embryos. Phenotypes were scored by microscopic observation and the diameter of the eyes was measured at 25 hpf. We classified three kinds of eye phenotypes by measuring the diameter of eyes: Diameter of normal eye = 140-150 μm, diameter of small eye <100 μm, diameter of large eye >150 μm. To reduce unknown defects, we discarded abnormally dividing embryos at 8- to 16-cell stages (1-5%).

*Dead embryos show a bustled phenotype after 18 hpf.

†Death by not clearly determined defects (data not shown).

‡Fz8a synergies with Wnt8b in a dose-dependent manner for dorsalizing effect.

§Fz8a-CRDTM acts in a dose-dependent manner for rescue of eye primordia.

n, total number of embryos analyzed.

binding domain (Fig. 4I) and acts exclusively as a repressor, was injected into embryos. Of all the dNTCF3 mRNA-injected embryos, 42.6% embryos (*n*=54) showed enlarged eye primordia (Fig. 4D,H) very similar to those seen in embryos injected with Fz8a-CRDTM mRNA (Fig. 4C,G). This similarity supports the conclusion that the effects of the dominant negative Fz8a construct are due to reduced activation of Wnt target genes.

Fz8a synergises with Wnt8b but not with Wnt1

wnt8b and *wnt1* are expressed in an overlapping pattern in the putative MHB starting from the late gastrula stage in zebrafish embryos (Kelly and Moon, 1995; Kelly et al., 1995). In order to ascertain whether Fz8a functionally interacts with Wnt8b or Wnt1 in vivo, we investigated the synergy between Fz8a and these two Wnts by examining their ability to dorsalise the embryo (ectopic dorsal axis induction or production of radialised embryos) and mimic phenotypes typically caused by much higher doses of *wnt8b* (Kelly et al., 1995). We injected an optimised amount of *wnt8b* (0.5-1 pg/embryo) and *fz8a* (5-20 pg/embryo) mRNAs; neither of these genes alone induces notochord expansion or secondary axis formation (Table 2). When *wnt8b* and *fz8a* mRNAs were co-injected into the one-cell embryos, a significant population of embryos exhibited dorsalised phenotypes (Table 2; Fig. 5A), including enlarged notochord (data not shown). In addition, a complete secondary axis formation was also observed in a small fraction of co-injected embryos (5.5%, *n*=200). The degree of dorsalisation was proportional to the amount of *fz8a* and *wnt8b* mRNAs injected, suggesting a functional interaction between Fz8a and Wnt8b (Table 2).

Ectopic expression of *wnt1* (5 pg/embryo) resulted in the embryos with loss of the anterior structures (100%, *n*=48) similar to that observed in embryos overexpressing *wnt8b*. However, co-injection of *wnt1* (5 pg/embryo) and *fz8a* (50 pg/embryo) mRNAs did not induce dorsalisation (0%, *n*=59, Fig. 5B).

A dominant-negative form of Fz8a can rescue the defects of anterior brain structure caused by the ectopic expression of Wnt8b

To provide additional evidence for an interaction between Fz8a and Wnt8b in generation of a posteriorizing signal during anterior brain patterning, we tested whether a dominant-negative form of Fz8a (Fz8a-CRDTM) could prevent the posteriorisation caused by *wnt8b* overexpression. When a low dose of *wnt8b* mRNA (0.5 pg/embryo) was injected, 61.1% of 25 hpf embryos (*n*=169) developed a small-eye phenotype, while a high dose of *wnt8b* mRNA (2 pg/embryo) induced an anterior brain defect together with a loss of the eye (90.6%, *n*=106; Table 2 and Fig. 5D). Co-injection of *fz8a-CRDTM* with *wnt8b* mRNA successfully rescued the loss of anterior brain structures caused by the overexpression of *wnt8b* mRNA (2 pg/embryo) in a dose-dependent manner (Table 2). As a result, 27.9% of the embryos (*n*=86) showed loss of eye (compare with 90.6% in *wnt8b* mRNA injection) when *fz8a-CRDTM* mRNA (20 pg/embryo) was co-injected with *wnt8b* mRNA; twice the amount of *fz8a-CRDTM* mRNA (40 pg/embryo) co-injected with *wnt8b* mRNA led to an almost complete recovery of the eye and forebrain (Fig. 5E,F). In accordance with these observations, *fz8a-CRDTM* mRNA, co-injected with *wnt8b* mRNA, almost restored *anf* (Fig. 5I), *rx3* (Fig. 5M,N) and *six3* (Fig. 5Q,R) expression, which disappeared in the anterior neuroectoderm of the 100% epiboly embryos overexpressing *wnt8b* (Fig. 5H,L,P). These results further support the suggestion that Fz8a and Wnt8b interact to provide a posteriorizing signal in the anterior neuroectoderm.

Although, as described in the previous section, no synergistic interaction was observed between Wnt1 and Fz8a, an interaction between this ligand and receptor cannot be ruled

out because co-injection of Fz8a-CRDTM mRNA with *wnt1* mRNA suppressed the posteriorisation caused by Wnt1 in a dose-dependent manner (data not shown), as shown in experiments with Wnt8b.

To demonstrate that Fz8a-CRDTM blocks posteriorisation defects caused by ectopic expression of Wnt1 and Wnt8b as direct consequence of its ability to interact with these ligands and not due to indirect effects on intracellular components of the Wnt signalling pathway, we showed that Fz8a-CRDTM cannot reverse posteriorisation caused by LiCl. One-cell stage embryos were injected with high dose of *fz8a-CRDTM* mRNA (300 pg/embryo), allowed to grow until the shield stage and exposed to LiCl for 15 minutes. In embryos injected with *fz8a-CRDTM* mRNA without LiCl treatment, an enlargement of anterior brain structures was observed. However, overexpression of Fz8a-CRDTM did not lead to a recovery of the eye and anterior brain structures lost after LiCl treatment (Fig. 5J). These results suggest that Fz8a-CRDTM is able to antagonise Wnt ligands, but it is unable to block the effects of LiCl, which leads to intracellular stabilisation of the Wnt signalling effector, β-catenin. In accordance with this observation, *fz8a-CRDTM* overexpression was unable to rescue the loss of anterior head structures in the *hdl* mutant (Kim et al., 2000), in which the repressor activity of Tcf3 for Wnt target genes was abolished by a null mutation (data not shown).

Knockdown of *fz8a* and *wnt8b* supports an important role for Fz8a-mediated Wnt8b signalling in anterior brain patterning

Recently, an antisense morpholino-based gene knockdown technique has enabled targeted loss-of-function studies in zebrafish embryos (Nasevicius and Ekker, 2000). To test directly whether Wnt8b and Fz8a are required for patterning the anterior brain, we generated gene-specific loss-of-function phenotypes for the two loci by injecting antisense morpholino oligonucleotides against *fz8a* (*fz8a*-MO) and *wnt8b* (*wnt8b*-MO) into the one-cell stage embryos (Fig. 6). The effects of *fz8a*-MO and *wnt8b*-MO on the anterior brain patterning were analysed at 100% epiboly with several anterior brain markers: *opl* for telencephalon and eye primordia, *six3* for eye primordia, *fkd5* for diencephalon (Varga et al., 1999), *eng2* for midbrain and *pax2* for MHB. The putative telencephalic and eye fate marked by *opl* was not affected by either gain- or loss-of-function of Wnt signal (Fig. 6A-D). By contrast, putative eye primordia, especially the posterior region of *six3* expression domain, was either expanded by *wnt8b*-MO (Fig. 6G) or slightly reduced by LiCl treatment at the late gastrula stage (Fig. 6H). However, no changes were seen in the eye primordia of embryos injected with *fz8a*-MO (Fig. 6F). These results suggest that while Wnt signalling mediated by Wnt8b at late gastrulation contributes to determining the size of the of eye primordia, Fz8a is not essential to transduce its effects and other Fz receptors may be involved. While the telencephalon and eye primordia markers were not noticeably affected by knockdown of *fz8a*, the posterior diencephalon and midbrain, marked by lateroposterior domain of *fkd5* and *eng2*, respectively, were significantly reduced in embryos injected with either *fz8a*-MO or *wnt8b*-MO (Fig. 6J,K,N,O). This

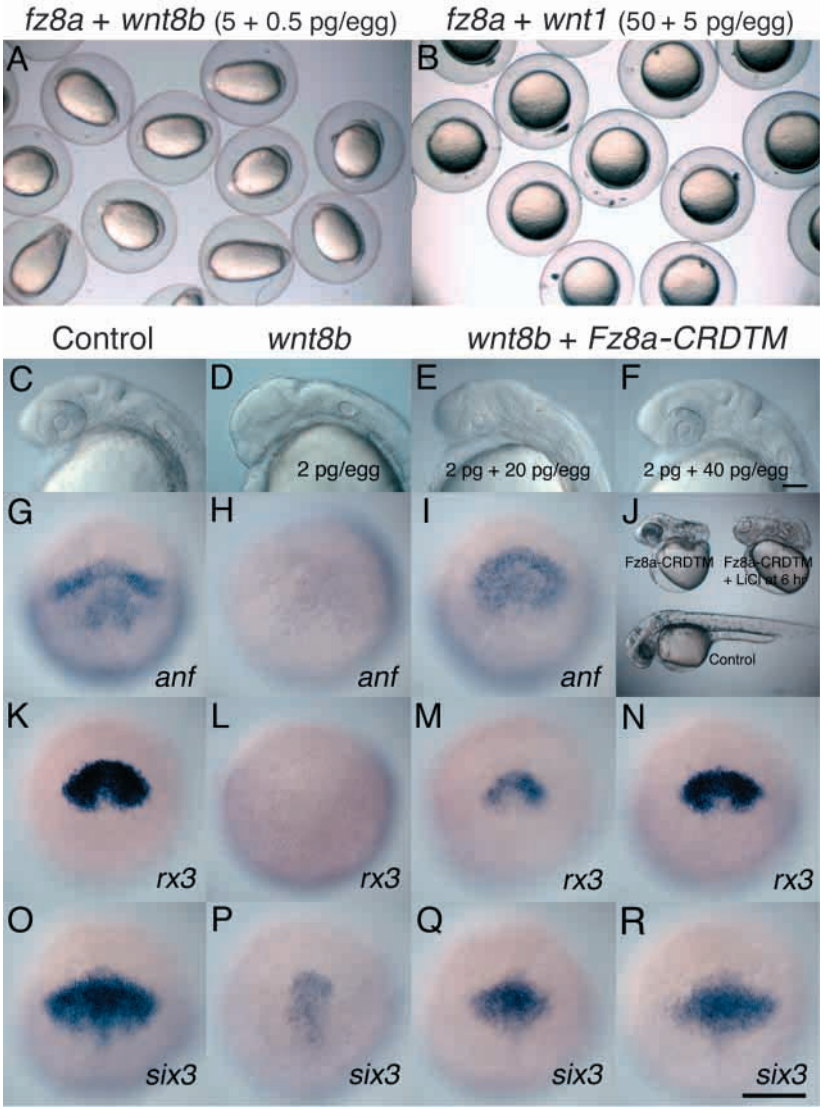

Fig. 5. Fz8a synergies with Wnt8b. The co-injection of *fz8a* (5 pg/embryo) with *wnt8b* (0.5 pg/embryo) causes radialised phenotype (A); however, co-injection of higher doses of *fz8a* (50 pg/embryo) with *wnt1* (5 pg/embryo) does not induce radialised phenotype (B). Dominant-negative form of Fz8a (*fz8a-CRDTM*) rescues loss of eye phenotype driven by *wnt8b* overexpression (D-F). 25 hpf embryos from uninjected control (C), *wnt8b* injection (D) and *wnt8b*/*fz8a-CRDTM* co-injection (E,F). Embryos were injected with *fz8a-CRDTM*, followed by either treatment with LiCl (or not) at the shield stage, and then further cultured to 27 hpf (J). The anterior brain defects caused by *wnt8b* overexpression are rescued by *fz8a-CRDTM* (G-I,K-R). The expression of *anf* (telencephalon and diencephalon), *rx3* (eye primordia) and *six3* (eye primordia), which disappeared at the putative anterior brain (H,L,P) of the 100% epiboly stage embryos, is restored by the co-injection of *fz8a-CRDTM* (I, M,N,Q,R). Scale bar: 100 μm in F; 250 μm in R.

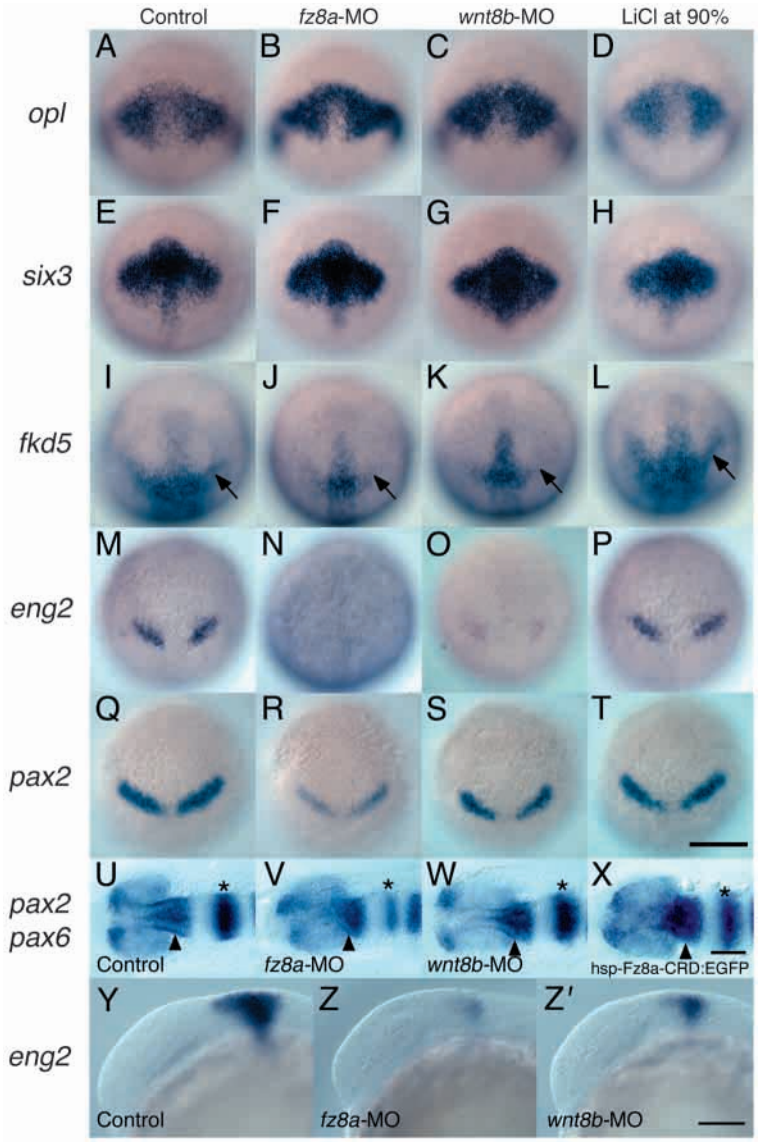

Fig. 6. Knockdown of *wnt8b* and *fz8a* alters the anterior brain patterning. The effects of morpholino oligonucleotides for *fz8a* (*fz8a*-MO) and *wnt8b* (*wnt8b*-MO), and LiCl treatment at late gastrula stage embryos on the expressions of anterior brain markers were analysed at the 100% epiboly stage (A-T). The putative telencephalon domain marked by *opl* is not affected by morpholinos (B, *fz8a*-MO; C, *wnt8b*-MO) or LiCl treatment (D). Note that caudal region of *six3* expression domain (eye primordia), which is not affected by *fz8a*-MO (F), is expanded posteriorly by *wnt8b*-MO (G). By contrast, LiCl treatment at late gastrula reduces *six3* expression (H). The posterior diencephalon marked by caudal domain of *fkd5* (arrow) is remarkably reduced by *fz8a*-MO (J) and *wnt8*b-MO (K), but LiCl treatment expands *fkd5* expression domain (L). The expression of *eng2* in midbrain almost disappears after treatment with *fz8a*-MO (N) and *wnt8b*-MO (O). However, LiCl treatment does not alter *eng2* expression in the putative midbrain domain (P). The putative MHB domain marked by *pax2* expression is reduced by *fz8a*-MO (R), although it is not affected by *wnt8b*-MO (S) or LiCl treatment (T). Scale bar: 250 μm. The effects of *fz8a*-MO, *wnt8b*-MO and *hsp-fz8a-CRD-EGFP* on the anterior brain patterning were analysed at the seven-somite stage embryos (U-Z′). The expression domains for *pax6* and *pax2* are remarkably reduced by *fz8a*-MO (V). However, *wnt8b*-MO injection reduces *pax6* expression domain without any changes of *pax2* expression domain (W). In embryos expressing a secreted dominant-negative form of Fz8a DNA under the heat-inducible promoter (*hsp-fz8a-CRD-EFGP*), *pax6* expression domain is reduced without any changes of *pax2* expression domain (X), similar to the embryos injected with *wnt8b*-MO (W). The expression domain for *eng2* is remarkably reduced by *fz8a*-MO (Z) or *wnt8b*-MO (Z′). Arrowheads and asterisks indicate *pax6* expression in the putative posterior diencephalon and *pax2* expression in MHB, respectively. Scale bars: in X, 125 μm for U-X; in Z′, 125 μm for Y-Z′; 250 μm for A-T.

observation indicates that Fz8a-mediated Wnt8b signalling is necessary for determining both posterior diencephalon and midbrain fates. By contrast, activation of Wnt signalling at the late gastrula by LiCl treatment expanded the posterior diencephalon (Fig. 6L), but the midbrain region was not affected by LiCl treatment (Fig. 6P). These results further suggest that putative midbrain region probably maintains relatively high activity of Fz8a-mediated Wnt8b signalling, while the posterior diencephalon region is likely exposed to the relatively low activity of Fz8a-mediated Wnt8b signalling during anterior brain patterning at late gastrula. Therefore, *eng2* expression in the midbrain might not be sensitively increased by the further activation of Wnt signalling driven by LiCl treatment, although it could be sensitively decreased by the reduction of Wnt signalling caused by either *fz8a*-MO or *wnt8b*-MO injection. By contrast, *fkd5* expression in the posterior diencephalon, where relatively low activity of *fz8a*-mediated *wnt8b* signalling probably persists, might be sensitive to both increased and decreased Wnt signalling. The expression level of *pax2* in MHB was partially decreased by *fz8a*-MO (Fig. 6R), although no changes were seen in embryos either injected with *wnt8b*-MO or treated with LiCl at the late gastrula stage (Fig. 6S,T). This observation further suggests that *pax2* expression in MHB fate is not regulated by Wnt8b signal, but is likely to be regulated by other earlier Wnt signal through Fz8a receptor during pre/early gastrulation.

To further confirm the role of Fz8a and Wnt8b in the anterior brain patterning, the effects of *fz8a*-MO and *wnt8b*-MO were also analysed at the seven-somite stage embryos (Fig. 6U-Z′). Injection of *fz8a*-MO reduced the posterior diencephalon domain expressing *pax6*, the MHB domain expressing *pax2* (Fig. 6V) and midbrain domain expressing *eng2* (Fig. 6Z), similar to the results seen in the 100% epiboly embryos (Fig. 6J,N,R). These results further support a role for Fz8a receptor in mediating Wnt signal during anterior brain patterning. Injection of *wnt8b*-MO induced slightly enlarged eye primordia, but reduced the posterior diencephalon domain expressing *pax6* without any changes of *pax2* expression domain in MHB of the seven-somite stage embryos (Fig. 6W). In addition, *wnt8b*-MO also significantly reduced *eng2*

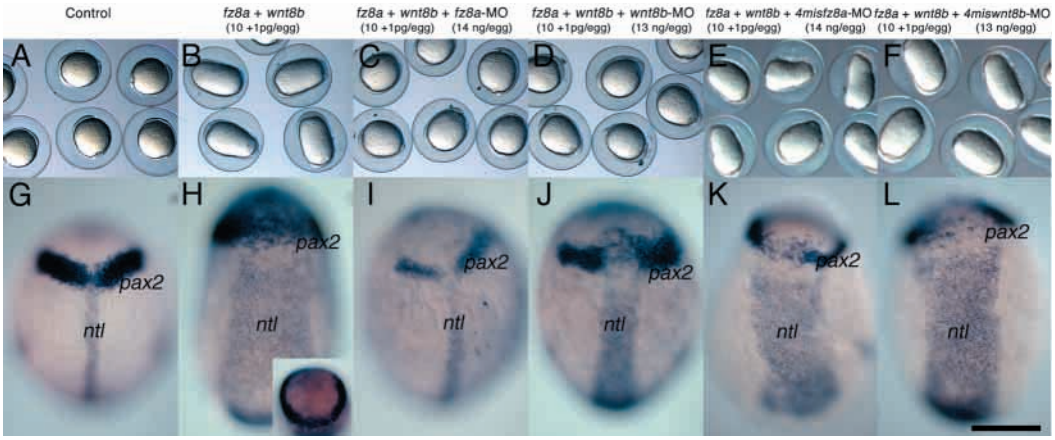

Fig. 7. Specificity of *fz8a*-MO and *wnt8b*-MO. The ability of *fz8a*-MO and *wnt8b*-MO, which can block dorsalisation induced by a co-injection of *fz8a* and *wnt8b*, were analysed at the bud stage embryos (A-D,G-J). Uninjected control embryos (A) and embryos dorsalised by a co-injection of *fz8a* (10 pg/embryo) and *wnt8b* (1 pg/embryo) mRNAs (B). Injection of *fz8a*-MO (14 ng/embryo) rescues the dorsalised phenotype induced by a co-injection of *fz8a* and *wnt8b* mRNAs (C). Injection of *wnt8b*-MO (13 ng/embryo) also rescues the dorsalised phenotype induced by a mixture of *fz8a* and *wnt8b* mRNAs (D). The expression of *pax2* and *no tail* (*ntl*) in uninjected control embryos (G). The radialised *pax2* expression and laterally expanded *ntl* expression caused by a co-injection of *fz8a* and *wnt8b* mRNAs (H) are rescued by *fz8a*-MO (I) and *wnt8b*-MO (J). The anterior view of the radialised expression of *pax2* is shown in an inset (H). In control morpholino experiment, neither *4misfz8a*-MO (E,F) nor *4miswnt8b*-MO (K,L) could rescue dorsalised phenotype induced by co-injection of *fz8a* and *wnt8b*. Scale bar: in L, 250 μm for G-L.

expression in the midbrain (Fig. 6Z′) as seen in the 100% epiboly stage (Fig. 6O).

As expression of *fz8a* starts from the MBT (Kim et al., 1988), changes in anterior brain patterning observed in embryos injected with *fz8a*-MO might be due to loss of its early function rather than its role at the late gastrula stage. Therefore, we analysed the effect of specifically inhibiting Fz8a function at the late gastrula stage on *pax2* and *pax6* expression in the anterior brain. A secreted dominant-negative form of Fz8a DNA with a heat-inducible promoter (*hsp-fz8a-CRD-EFGP*) was injected into embryos and its expression was induced from 75% epiboly by heat-shock at 37°C for 1 hour, and then embryos were returned to 28.5°C. Embryos with higher EGFP expression were selected (data not shown), further cultured to the seven-somite stage, and changes in *pax2* and *pax6* expression in the anterior brain were analysed. As a result, *hsp-fz8a-CRD-EFGP* induced a similar phenotype to that seen in embryos injected with *wnt8b*-MO (Fig. 6W,X).

This result further supports our data that Fz8a-mediated Wnt8b signalling during late gastrulation plays an important role in regulation of the anterior brain patterning.

Specificity of *fz8a*-MO and *wnt8b*-MO

Previously, we have shown that co-injection of *fz8a* and *wnt8b* can synergistically produce a dorsalised phenotype at doses where neither *fz8a* nor *wnt8b* alone would have this effect (Table 2). Based on this observation, we predicted that inhibiting the function of either *fz8a* or *wnt8b* with morpholinos would prevent the dorsalised phenotype. To reveal the specificity in the effects of *fz8a*-MO and *wnt8b*-MO, either morpholino was co-injected with a mixture of *fz8a* and *wnt8b* mRNAs into yolk of the one-cell stage embryos (Fig. 7). As before, embryos co-injected with a mixture of *fz8a* and *wnt8b*

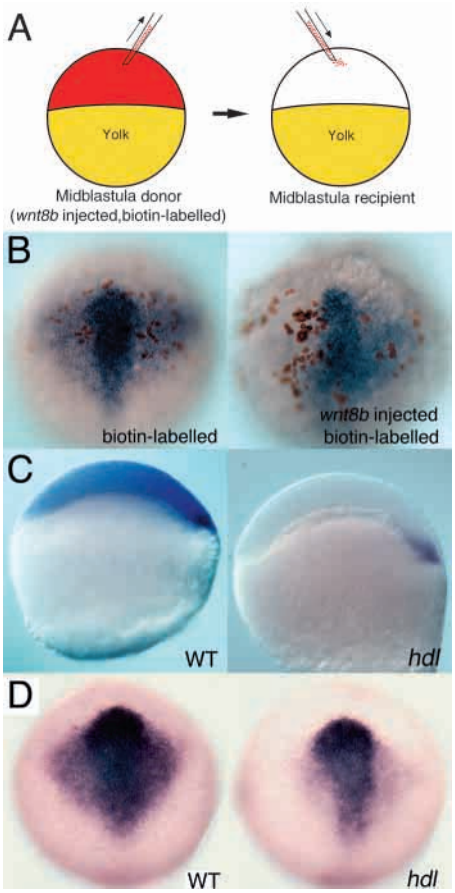

Fig. 8. The expression of *fz8a* is negatively regulated by Wnt8b. (A) Schematic representation of the transplantation experiment. A small group of 5-10 cells that overexpresses *wnt8b* was drawn up from a labelled midblastula donor embryo and transplanted into the blastoderm of the midblastula host embryos. (B) The control donor cells cannot affect *fz8a* expression in the anterior neuroectoderm of the recipient embryo (left), while transplanted cells that overexpress *wnt8b* strongly suppress *fz8a* expression in the anterior neuroectoderm of the 100% epiboly stage of host embryos (right). The transplanted embryonic cells are visualised in brown. The expression of *fz8a* in wild type (WT) (left) and *headless* (*hdl*) mutant (right) at the 30% epiboly stage (C) and 100% epiboly stage (D). In *hdl* mutant embryos, *fz8a* expression in the neuroectoderm is remarkably decreased.

mRNAs showed a dorsalised phenotype; the axial mesoderm marked by *ntl* expression was laterally expanded and *pax2* expression domain was radialised (Fig. 7B,H). By contrast, co-injection of *fz8a*-MO with the mixture of *fz8a* and *wnt8b* mRNAs prevented or reduced the dorsalised phenotype (Fig. 7C,I). Similarly, the *wnt8b*-MO reduced the dorsalised phenotype of embryos injected with *fz8a* and *wnt8b* mRNAs (Fig. 7D,J). To further confirm the specificity of *fz8a*-MO and *wnt8b*-MO, we designed four base pair mismatched *fz8a*-MO (*4misfz8a*-MO) and *wnt8b*-MO (*4miswnt8b*-MO). Neither *4misfz8a*-MO nor *4miswnt8b*-MO could rescue dorsalised phenotype induced by co-injection of *fz8a and wnt8b* (Fig. 7E,F,K,L). In addition, we co-injected *wnt8b*-MO (9 ng/embryo) with *wnt8b* mRNA (2 pg/embryo) into the one-cell stage embryos. The co-injection successfully rescued the eye defects (eyeless or small eye phenotype) caused by *wnt8b* mRNA (92%, *n*=36; data not shown). These results, taken together, suggest *fz8a*-MO and *wnt8b*-MO specifically knockdown function of *fz8a* and *wnt8b*, respectively.

Wnt8b can suppress *fz8a* expression in the anterior neuroectoderm

By the 100% epiboly stage, the expression domains for *fz8a* and *wnt8b* in the anterior neuroectoderm were separated by a gap of two to three cell diameters, though expression domains of *fz8a* and *wnt8b* partially overlapped at 90% epiboly stage (Fig. 1). Furthermore, a decreasing gradient of *fz8a* expression from rostral to caudal was noticed in the anterior neuroectoderm by the bud stage embryos (data not shown). This observation raised the possibility that Wnt8b might suppress *fz8a* expression in the anterior neuroectoderm just like *wingless* (*wg*) suppresses *Dfz2* expression in *Drosophila* (Cadigan et al., 1998). To test this hypothesis, we transplanted cells from a donor (injected with *wnt8b* RNA at the one cells stage) into wild-type embryos at the mid-blastula stage (Fig. 8A). In the late gastrula of wild-type host embryos, *fz8a* mRNA transcripts almost disappeared from the anterior neuroectoderm near the transplanted cells, indicating that Wnt8b signalling suppresses *fz8a* expression in the anterior neuroectoderm (Fig. 8B). This suppression of *fz8a* by Wnt signalling was further confirmed in *hdl* mutant that are characterised by exaggerated Wnt signalling. As expected, *fz8a* expression in the blastoderm of the 30% epiboly stage as well as the anterior neuroectoderm of bud stage *hdl* mutant embryos was almost completely lost (Fig. 8C,D). Consistent with this suppression of *fz8a* by Wnt signalling, injection of *fz8a-CRDTM* or *dNTCF3* mRNAs resulted in an increase of *fz8a* expression in the anterior neuroectoderm (data not shown). Taken together, these results indicate that *fz8a* expression in the anterior neuroectoderm is negatively regulated by Wnt8b signalling.

DISCUSSION

During vertebrate development, head induction requires the blockage of posteriorizing Wnt activity. The three potent Wnt antagonists, Cerberus, Frzb1 and Dickkopf 1 (Dkk1), produced in endoderm and mesoderm have been reported to play critical roles in head induction (Bouwmeester et al., 1996; Leyns et al., 1997; Wang et al., 1997; Glinka et al., 1998). However, the molecular mechanism responsible for defining anterior neuroectodermal fates into more subdivided brain territories, such as telencephalon, retina, diencephalon, midbrain and MHB, during gastrulation has not been well established, although important roles of *eng2*/*pax2* and *pax6* in the determination of forebrain/midbrain boundary during the segmentation stage have been reported in mice and zebrafish (Araki and Nakamura, 1999; Matsunaga et al., 2000).

While this paper was in revision, two papers provided evidences that rostrocaudal brain characters can be specified by Wnt3A signal in a dose-dependent manner (Kieker and Niehrs, 2001; Nordstrom et al., 2002); an artificially generated Wnt gradient activity helps subdivision of the neuroectodermal field into various brain regions including the telencephalon, diencephalon, midbrain, MHB and hindbrain. However, our data show that the posteriorizing activity of Fz8a-mediated Wnt8b signalling in vivo at the late gastrula stage defines domains for the posterior diencephalon and midbrain. Our study thus provides the first evidence that Fz8a-mediated Wnt8b signalling at the late gastrula stage is necessary for proper patterning of the anterior brain.

A Wnt antagonist for head formation is required before mid-gastrula, but anterior brain patterning requires Wnt signalling at late gastrula

In previous studies, increased activity of a Wnts signal after MBT resulted in the loss of forebrain structures: increase of Wnt signal activity by injection of Wnt mRNA, LiCl or CMV promoter-driven *wnt8* or *wnt8b* plasmids resulted in the loss of anterior neural fates, including the diencephalon (Saint-Jeannet et al., 1997; Fredieu et al., 1997; McGrew et al., 1995; Kelly et al., 1995; Chang and Hemmati-Brivanlou, 1998; Popperl et al., 1997). However, our LiCl treatment studies demonstrate that stimulation of Wnt signalling from the late gastrula onwards no longer has a loss of anterior brain structures, but instead result in transformation of anterior fate into more posterior one within anterior brain region. These observations suggest that Wnt antagonists expressed in mesendoderm (Niehrs, 1999) required for head induction might act at a relatively early stage, before cell fates are determined in the anterior neuroectoderm (which is likely to occur around the late gastrula stage).

Wnt8b-mediated repression of *fz8a* expression might control short- and long-range Wnt8b gradient in the anterior neuroectoderm

Our results demonstrate that Fz8a-mediated Wnt8b signalling is needed for the maintenance of proper forebrain territory, as well as specification of the midbrain. However, the presumptive forebrain, including posterior diencephalon, is far from the presumptive midbrain, where *wnt8b* is expressed. Therefore, additional explanation is required for the mechanism of long-range transfer of unstable Wnt8b to the forebrain region from midbrain.

In *Drosophila* wing development, the activity of highly unstable Wg (the *Drosophila* homologue of Wnt) protein alone rapidly decreases by diffusing a few cell diameters from the site of Wg expression (Couso et al., 1994; Neumann and Cohen, 1997a). However, the Wg morphogen is stabilised by binding to its receptor Dfz2, thus enabling Wg protein to migrate longer distances (at least 50 μm) from the region

expressing Wg (Zecca et al., 1996; Neumann and Cohen, 1997b; Cadigan et al., 1998). In addition, Wg is defined as a localised factor that controls its own range of action by repressing the expression of a Dfz2 receptor that stabilises it during wing development (Cadigan et al., 1998). By these mechanisms, Wg can differentially regulate its short-range (higher activity of Wnt signal) and long-range (lower activity of Wnt signal) target genes during wing development.

In the anterior neuroectoderm of the zebrafish late gastrula, *fz8a* and *wnt8b* are expressed adjacent to each other. Therefore, it is expected that Wnt8b also can travel a longer distance from its site of synthesis by binding with Fz8a as seen in *Drosophila*. Furthermore, in our study, the transplanted cells that overexpress *wnt8b* suppressed *fz8a* expression in the anterior neuroectoderm of the recipient embryos. These observations suggest that the long-range and short-range transfer of Wnt signalling evidenced in *Drosophila* wing development might also be conserved in zebrafish anterior brain patterning, although the in vivo data that reveal the diffusion of Wnt8b through binding with Fz8a in zebrafish is not available yet. We therefore suggest as a possible mechanism that the short-range and long-range transfer of Fz8a-mediated Wnt signalling can define the midbrain and forebrain territory, respectively.

Expression of *pax6* in the posterior diencephalon might be a consequence of *anf* repression caused by Fz8a-mediated Wnt8b signalling

In *Xenopus*, *pax6* transcripts distributed at the anterior neuroectoderm are known to be under the inhibitory control of *anf*. The expression of *anf* is initially detected in the entire presumptive diencephalon and then eventually disappears except in the future ventral diencephalic cells of the midline at the late gastrula stage (Ermakova et al., 1999; Kazanskaya et al., 1997). Our data show that activation of Wnt signalling by LiCl treatment or *wnt8b* overexpression completely abolishes *anf* expression in the anterior neuroectoderm, but expands the posterior diencephalon region marked by *pax6* expression. Therefore, this observation suggests that *pax6* expression in the posterior diencephalon might be indirectly activated by the long-range action of Fz8a-mediated Wnt8b signalling. Instead, the suppression of *anf* expression accomplished by long-range action of Fz8a-mediated Wnt8b signalling probably contributes to the upregulation of *pax6* expression in the posterior diencephalon.

Possible involvement of multiple Fz and Wnt interactions in anterior brain patterning

In morpholino experiment, *eng2* expression in the midbrain was significantly reduced by either *fz8a*-MO or *wnt8b*-MO injection, thus indicating that *eng2* expression requires both Fz8a and Wnt8b. By contrast, putative eye primordia, marked by *six3*, were expanded by *wnt8b*-MO, but no changes were induced in *six3* expression domain by *fz8a*-MO. These results suggest that other Fz receptors probably mediate Wnt8b signal during fate determination of the eye primordia, although other *fz* genes expressed in the presumptive eye primordia at late gastrulation have not been identified.

However, *pax2* expression in MHB, which was not reduced by *wnt8b*-MO but downregulated by *fz8a*-MO, raised a possibility that other earlier Wnt signals occur at pre/early gastrulation might regulate *pax2* expression in the putative MHB through Fz8a receptor. Recently, two Wnt8 isoforms produced by a bicistronic transcript were reported to be required for proper initiation of *pax2* expression in the presumptive MHB (Lekven et al., 2001). Thus, a possibility of *fz8a* and Wnt8 interaction in regulating *pax2* expression in MHB still remains to be elucidated. Alternatively, we can not rule out the possible contribution of Fz7 and Wnt8 interaction for regulating *pax2* expression in MHB, as *fz7* expression was also reported in the presumptive MHB of zebrafish late gastrula embryos (El-Messaoudi and Renucci, 2001). Therefore, further studies are needed to address the detailed functions of multiple Fz and Wnt interactions in regulating fate determination of the eye primordia and MHB.

A gradient of Fz8a-mediated Wnt8b signal activity patterns the posterior diencephalon and midbrain

Our data suggest that LiCl treatment at the late gastrula stage (90% epiboly) acts as an artificial Wnt signal activator, thus significantly increasing *fkd5* and *pax6* expression in the posterior diencephalon. However, *eng2* expression was not dramatically increased, although Wnt signalling was highly activated by LiCl treatment at the late gastrula stage. Nevertheless, injections of *wnt8b*-MO and *fz8a*-MO morpholinos, which might cause partial reductions of Wnt8b and Fz8a, reduced *eng2* expression in the midbrain more sharply compared with decreased expressions of *fkd5* and *pax6* in the posterior diencephalon. These results indicate that *eng2* in the midbrain is highly sensitive to a decrease of Wnt8b signal activity but less sensitive to an excess of Wnt signal, whereas *fkd5* and *pax6* in the posterior diencephalon is highly sensitive to an excess of Wnt signal but less sensitive to a decrease of Wnt8b signal. These observations indicate that patterning of the midbrain needs a higher threshold of Wnt8b activity, while that of the posterior diencephalon may require relatively lower Wnt8b thresholds.

To explain a gradient of Fz8a-mediated Wnt8b signal activity required for the proper patterning of the anterior neuroectoderm (posterior diencephalon and midbrain), we propose a model that can generate a sharp gradient of Fz8a-mediated Wnt8b signalling activity, with a peak at the midbrain (Fig. 9). First, at the 90% epiboly stage, two adjust expression domains for *fz8a* and *wnt8b* are partially overlapped in the putative midbrain. At the same time, a small amount of Wnt8b, possibly stabilised by binding to Fz8a, might further diffuse towards the presumptive posterior diencephalon from midbrain. Therefore, low Wnt8b signal activity and high Wnt8b signal activity might be imposed on the posterior diencephalon and midbrain region, respectively. Subsequently, at late gastrula stage, overlapping two expression domains are separated by the repression of *fz8a* expression caused by Wnt8b with generating a decreasing gradient of Fz8a receptor towards the caudal anterior neuroectoderm, thus a gradient of Fz8a-mediated Wnt8b signal activity becomes sharper at late gastrula stage. Consequently, a gradient of *pax6* expression in the diencephalon from posterior to anterior can be established by low level of Wnt8b activity, while *eng2* expression in the midbrain can be regulated by high level of Wnt8b activity. This hypothesis that *pax6* and *eng2* expression requires lower and higher level of Wnt signalling, respectively, was recently evidenced in chick gastrula (Nordstrom et al., 2002) during revision of this paper.

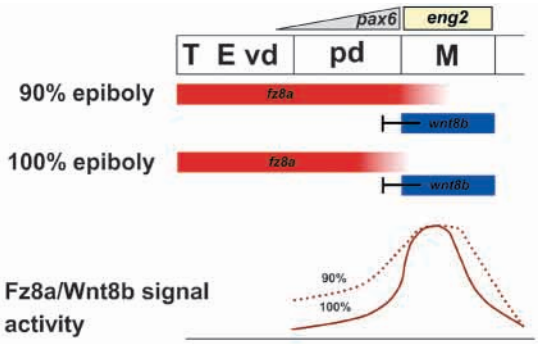

Fig. 9. A proposed mechanism can generate a differential gradient of Fz8a-mediated Wnt8b signalling required for the anterior brain patterning. The spatial expression pattern of *fz8a*, *wnt8b*, *pax6* and *eng2* in the presumptive forebrain and midbrain domains of the 90% and 100% epiboly stage embryos is schematically illustrated. At the 90% epiboly stage, the posterior expression domain of *fz8a* overlaps that of *wnt8b* expression. At the 100% epiboly stage, *fz8a* expression within this overlapping domain gradually lost its expression by the repression Wnt8b that is secreted from presumptive midbrain. This repression might decrease Fz8a/Wnt8b signal activity in the presumptive posterior diencephalon, thus generating a sharp gradient of Fz8a/Wnt8b signal activity at 100% epiboly stage. As results, *eng2* (light yellow) expression was probably induced by higher Fz8a/Wnt8b signal activity in the presumptive midbrain during late gastrulation. However, anteriorly transferred Wnt8b can establish lower Fz8a/Wnt8b signalling activity, thus defines territory of *pax6* expression in the presumptive diencephalon (grey). Broken and unbroken brown lines indicate hypothetical gradient of Fz8a/Wnt8b signal activity at the 90% and 100% epiboly stage, respectively. T, telencephalon; E, eye primordium; vd, ventral diencephalon; M, midbrain.

We conclude that the Wnt signal activity accomplished by the functional interaction between Wnt8b and Fz8a within the anterior neuroectoderm is required for zebrafish anterior brain patterning. In addition, we also propose that a gradient of Fz8a-mediated Wnt8b signalling with a peak in the midbrain might play an important role in the fate determination of the anterior brain.

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