Int. J. Dev. Biol. 47: 245-252 (2003)

Original Article

Flamingo, a cadherin-type receptor involved in the *Drosophila* planar polarity pathway, can block signaling via the canonical wnt pathway in *Xenopus laevis*

RICHARD MORGAN*, ALI-MORSI EL-KADI and CHRISTOPHER THEOKLI

Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, London U.K.

ABSTRACT The *Flamingo* gene encodes a seven-pass transmembrane receptor of the cadherin super family and is one of a growing number of components identified as being necessary for the establishment of planar polarity in the *Drosophila* wing. Although vertebrate homologues of *Flamingo* have been identified in both man and mice, no function has as yet been ascribed to them. Here, we report the cloning of the *Xenopus* homologue of *Flamingo (XFmi). XFmi* is expressed in the dorsal ectoderm during gastrulation and in the forebrain and midbrain subsequently. We show that ectopic expression of the murine *Flamingo* gene can prevent the wnt mediated posteriorisation of the neural plate by interfering with the canonical wnt signalling pathway.

KEY WORDS: *Flamingo gene, Xenopus laevis, early development, canonical wnt pathway, Hox, neural patterning*

Introduction

It is becoming increasingly common to find that a component of one cell signalling pathway is used in a second, distinct pathway and therefore participates in two distinct cellular or developmental processes. One such example is the product of the *Flamingo* (*Fmi*) gene, a seven-pass transmembrane receptor of the cadherin super family (Usui *et al.,* 1999). *Fmi* was originally identified in *Drosophila* as being required for the correct planar polarity of sensory bristles and asymmetric cell division in the epidermis (Lu *et al.,* 1999; Chae *et al.,* 1999; Usui *et al.,* 1999). These functions depend on the localisation of FMI protein to the proximal / distal cell boundary, a process which in turn requires the contribution of the *Frizzled* (*Fz*) and *Dishevelled* (*Dsh*) genes (Lu *et al.,* 1999; Chae *et al.,* 1999; Usui *et al.,* 1999; Shimada *et al.,* 2001; reviewed by Adler and Lee, 2001). Together, FMI, FZ and DSH proteins may form a complex that directs the localised reorganisation of the cytoskeleton to form a hair at the distal edge of the cell (Shimada *et al.,* 2001).

A second function of *Fmi* is in the correct control of dendrite branching. Neurones elaborate dendrites with highly stereotyped branching patterns, which may arise from intrinsic properties of the neurone or from competition between neighbours, whereby the dendrites of homologous neurones repel each other. This repulsion requires *Fmi*, but not *Fz* (Gao *et al.,* 1999; Gao *et al.,* 2000). Hence *Fmi* functions in two separate cellular signalling pathways, each mediating different developmental responses.

Although the vertebrate homologue of *Fmi* has now been cloned in mice and humans, to date there is no indication as to what its function may be in early development. Here we report the cloning of the *Xenopus* homologue of the *Fmi* gene *(XFmi). XFmi* is expressed in the presumptive anterior neural plate during gastrulation and can prevent the wnt-mediated posteriorisation of this tissue.

Results

XFmi *has a Very High Level of Sequence Identity to the Mouse* Flamingo1 *Gene*

In order to look for downstream targets of the *Xenopus Hoxb4* gene (*XHoxb4*), we used a differential display technique to compare gene expression in control embryos with those over expressing *XHoxb4*. A number of cDNAs were significantly under-represented in *XHoxb4* over-expressing embryos, and one of these was found to have very high (63%) degree of sequence identity to the mouse *Flamingo1* gene (Usui *et al.,* 1999), a homologue of the *Drosophila Flamingo* gene (also known as *starry night*, Chae *et al.,* 1999). On the basis of this very high degree of sequence identity we concluded

Abbreviations used in this paper: XFmi, Xenopus homologue of Flamingo gene.

***Address correspondence to:** Dr. Richard Morgan. Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K. Fax: +44-208-725-3326. e-mail: rmorgan@sghms.ac.uk

0214-6282/2003/$25.00

Printed in Spain
www.ijdb.ehu.es

that the isolated cDNA is in fact a partial clone of the *Xenopus* homologue of *Flamingo*, and we therefore named it *XFlamingo* (*XFmi*; GenBank accession number AF518403).

XFmi *Transcripts are present Maternally but disappear during Neuralation, after which* XFmi *is strongly expressed in the Brain and Anterior Neural Tube*

In order to examine *XFmi* expression, we used RT-PCR to assess the relative amount of *XFmi* transcripts present in RNA extracted from embryos at different developmental stages (Fig. 1). *XFmi* is present maternally (i.e. before the onset of zygotic transcription (Newport and Kirschner, 1982)), and decreases throughout gastrulation to such an extent that transcripts cannot be detected during neurulation. Strong *XFmi* expression is detected subsequently however, at the early tail bud stage.

We used whole mount *in situ* hybridisation to study the spatial distribution of *XFmi* transcripts during early development (Fig. 2). In agreement with the data in Fig. 1, *XFmi* is expressed during gastrulation, although the signal is faint. At this stage (10+) it is confined to those cells that have involuted through the dorsal side of the blastopore and the ectodermal cells that overlie them (the presumptive neural plate; Fig. 2 A,B). *XFmi* can not be detected at early neurula stages (Fig. 2C), but staining is visible again in the anterior end of the embryo by the mid neurula stages (Fig. 2D). Its expression becomes far stronger at the end of neurulation and by the tail bud stage there is a strong signal in the eyes and forebrain (Fig. 2 E-J). Expression becomes progressively weaker posteriorly and cannot be detected beyond the hindbrain / spinal chord boundary (Fig. 2 E,F).

Ectopic Expression of MFmi in Whole Embryos causes an Expansion of the Otx2 Expression Domain

The restricted expression of *XFmi* in development indicates that it might have a regulatory role in those cells that express it. In order to understand what this might be, ectopic expression of *Fmi* was induced in embryos by injecting 50 pg of full length mouse *Flamingo 1* (*MFmi*) RNA into each cell of the four cell stage embryo. The majority of embryos treated in this way do not complete gastrulation; their blastopores fail to close and they die before their untreated counterparts reach the early neural stage (13). In order to asses possible affects of *MFmi* overexpression on patterning at these early stages, the expression of a number of genes was examined by *in situ* hybridization analysis. Of these, the expression of *Otx2* is considerably greater in *MFmi* injected embryos, as opposed to control embryos injected with *MFmi* RNA lacking a translation start site (Fig. 3). *MFmi* RNA lacking this site is not translated to give MFMI protein (verified by *in vitro* translation, data not shown). *Otx2* is a marker of the anterior most structures in the embryo, including the anterior neural plate (that gives rise to the forebrain and mid brain; Pannese *et al.*, 1995; Blitz and Cho, 1995).

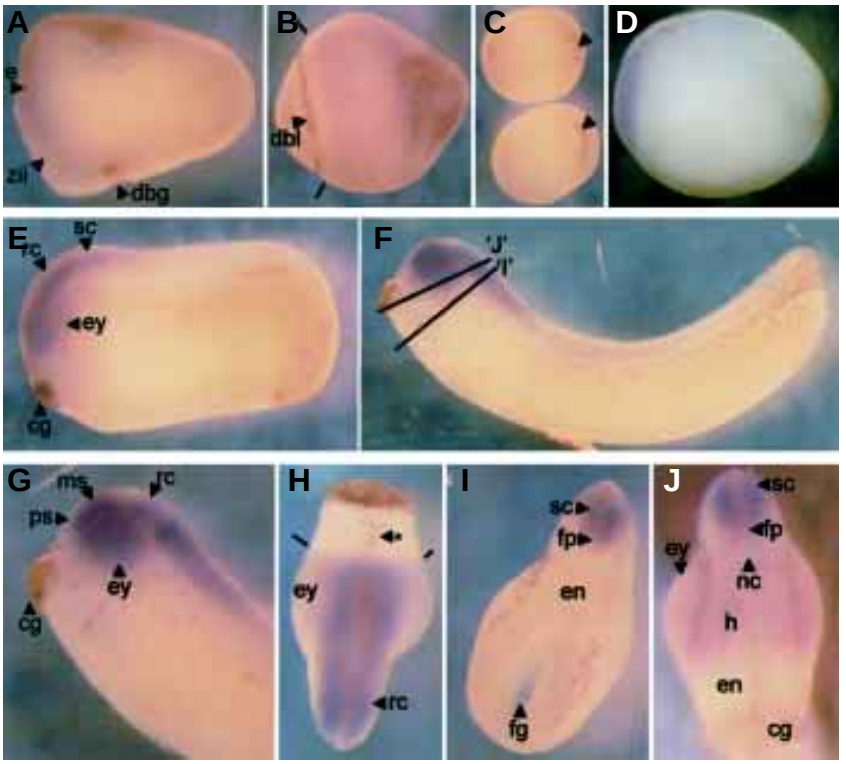

Fig. 2. *In situ* hybridisation analysis of *XFmi* expression in whole embryos. (A) *Sagittal section through an early gastrula (stage 10+) embryo. Faint expression of* XFmi *can be seen in the zone of internal involution just above the dorsal blastopore groove, and also in the ectodermal cells closer to the animal pole (the vegetal pole of the embryo is at the bottom, the animal pole at the top).* **(B)** *Dorsal view of the same stage embryo (10+).* XFmi *expression begins at a defined border above the dorsal blastopore lip (marked by the two lines), corresponding to the involuted cells.* **(C)** *Dorsal view of two early neurula (stage 13) embryos, the posterior end (marked by yolk plug; arrow heads) is on the right. No expression is detectable at this stage.* **(D)** *Lateral view of a mid neurula (stage 17) embryo, dorsal side uppermost. A faint staining is apparent at the anterior end of the embryo.* **(E)** *Lateral view of an early tail bud (stage 22) embryo, anterior to left.* **(F)** *Lateral view of a tail bud (stage 26) embryo, anterior to left.* **(G)** *Enlargement of the head of the embryo shown in (E).* **(H)** *Dorsal - anterior view of the embryo shown in (F), with the anterior-most end upwards. The anterior limit of* XFmi *expression is indicated by the two lines. A group of cells scattered around the midline, and anterior to this line, also express* XFmi *and are indicated by *.* **(I)** *Section through the embryo shown in (F) across indicated plane ('I'). The dorsal side is at the top.* **(J)** *Section through the embryo shown in (F) across the indicated plane ('J'). The dorsal side is at the top. Abbreviations: cg, cement gland; dbg, dorsal blastopore grove; dbl, dorsal blastopore lip; e, ectoderm; en, endoderm; ey, eye; fg, foregut; fp, floor plate; h, head mesenchyme; ms, mesencephalon; nc, notochord; ps, prosencephalon; rc, rhombencephalon; sc, spinal chord; zii, zone of internal involution.*

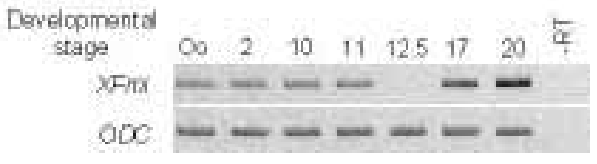

Fig. 1. *XFmi* expression during early *Xenopus* development. *RT-PCR analysis of* XFmi *expression in embryos of different developmental stages, as shown. Oo, oocyte. Stage 2 is a 4 cell embryo; 10, early gastrula; 11, mid gastrula; 12.5, late gastrula / early neurula; 17, late neurula; 20, early tail bud. ODC, ornithine decarboxylase (loading control).*

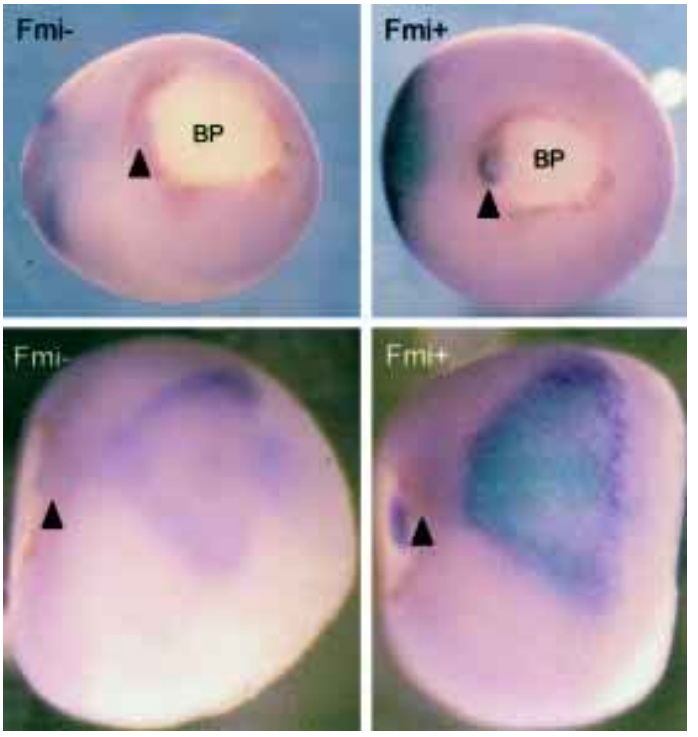

Fig. 3. Ectopic expression of *MFmi* results in the anteriorisation of the early embryo. *Fertilised* Xenopus *eggs were injected with* MFmi *RNA (Fmi+), or with a control transcript lacking the translation start site (Fmi-). Embryos were allowed to develop to the late gastrula stage (11.5) and analyzed for the expression of* Otx2 *by* in situ *hybridization (blue / purple staining). The embryos are viewed with the blastopore (BP) uppermost in the top two panels, and with the blastopore to the left in the bottom two. The arrow head marks the position of the dorsal blastopore lip.*

MFmi Expression in Neural Ectoderm prevents Posteriorisation by Wnt3A but not by β-Catenin

The previously reported association of *Flamingo* with the frizzled protein (a receptor for the wnt ligand in the canonical wnt signalling pathway), coupled with the observed expression of *XFmi* in the anterior neural tube and the up-regulation of *Otx2* in embryos ectopically expressing *MFmi*, lead us to look at whether *Fmi* might influence wnt mediated posteriorisation of the neural plate. This is a classic assay for wnt activity in *Xenopus*, and involves over expressing wnt in naive ectodermal explants (Domingos *et al.,* 2001; McGrew *et al.,* 1995; Yamaguchi, 2001). To achieve this fertilised eggs were injected with *XWnt3A*, *MFmi*, *Noggin* and a number of other mRNAs, in various combinations (Fig. 4A). The eggs were then allowed to develop to the early gastrula stage (10), whereupon the ectodermal cells over the blastocoel were removed (the 'cap'). These were cultured until their untreated siblings had reached the early tailbud stage, at which point total RNA was extracted and analysed for the expression of specific marker genes (Fig. 4B).

Caps from untreated embryos do not express neural markers (Fig. 4B), but when the neural inducer *noggin* is injected caps subsequently express *noggin* and adopt an anterior neural fate (Lamb *et al.,* 1993). Hence caps from *noggin* injected embryos express the pan-neural marker *NCAM* (Balak *et al.,* 1987) along with *Otx2*, a transcription factor that is only expressed in the anterior most part of the neural tube (the forebrain and mid brain; Pannese *et al.,* 1995; Blitz and Cho, 1995). When *Wnt3A* and *noggin* are both present in caps the resulting neural tissue is more posterior in character. The expression of *Otx2* is repressed, whilst the *Hox* genes *XHoxb1*, *XHoxb4* and *XHoxb9* are all upregulated (Domingos *et al.,* 2001; McGrew *et al.,* 1995; Fig. 4B). These genes have anterior limits of expression considerably posterior to *XOtx2*, being the rhombomere 2/3 and rhombomere 6/7 boundaries for *XHoxb1* and *XHoxb4*, respectively, and in the spinal chord for *XHoxb9* (Godsave *et al.,* 1994).

When the mouse homologue of *Flamingo* (*MFmi*) is co-injected with *noggin* RNA there is no perceivable difference in the expression of the marker genes to that seen when noggin alone is injected. However, when *MFmi* is present with both *noggin* and *Wnt3A* there is a significant reduction in the expression of the posterior markers, with *XHoxb9* expression being reduced to a level that cannot be detected by RT-PCR. This effect can be reversed by adding an antisense 'morpholino' that is complementary to the translation start site on *MFmi* and hence can block *MFmi* translation. Morpholinos are chemically modified oligonucleotides that have been shown to have both increased stability and significantly lower toxicity than unmodified oligos in *Xenopus* embryos (Heasman *et al.,* 2000; Ross *et al.,*

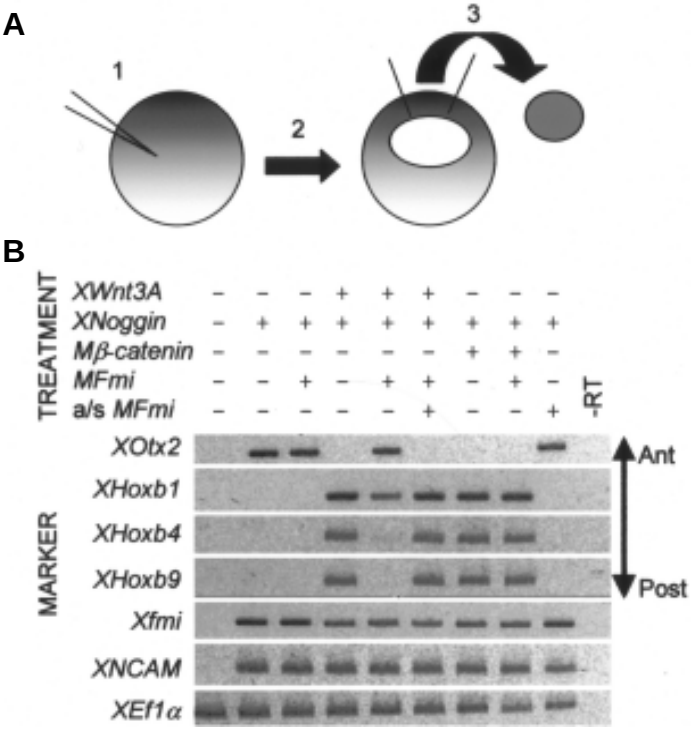

Fig. 4. *Fmi* can block the posteriorisation of neural tissue by *Wnt3A*. (A) *Schematic overview of the experimental procedure. Fertilised* Xenopus *eggs were injected with various combinations of RNAs (1) and cultured until the early gastrula stage (2). At this point the animal cap was removed (3) and cultured until sibling embryos had reached the early tail bud stage. Total RNA was extracted and analysed for marker gene expression by RT-PCR.* **(B)** *RT-PCR analysis of caps expressing a combination of different injected RNAs, as shown ('TREATMENT'). The expression of various marker genes was analysed by RT-PCR ('MARKER'). a/s MFmi, morpholino oligo with complementary sequence to the translation start site of the* MFmi *RNA.* XOtx2, XHoxb1, XHoxb4 *and* XHoxb9 *are arranged in anterior (Ant) to posterior (Post) order, as indicated by the double headed arrow.*

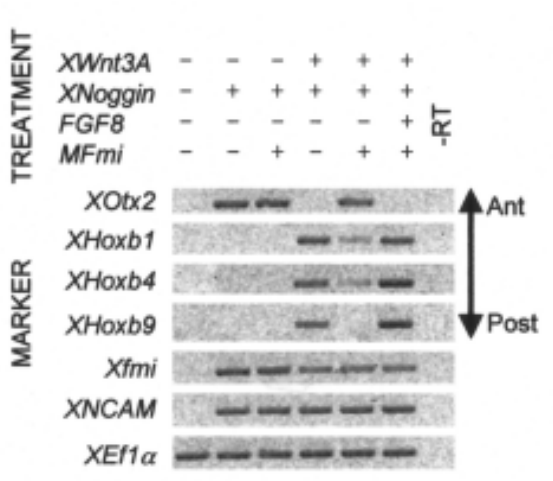

Fig. 5. Flamingo does not block the FGF mediated posteriorisation of the neural plate. *RT-PCR analysis of caps expressing a combination of different injected RNAs, as shown ('TREATMENT'). The expression of various marker genes was analysed by RT-PCR ('MARKER').* XOtx2, XHoxb1, XHoxb4 *and* XHoxb9 *are arranged in anterior (Ant) to posterior (Post) order, as indicated by the double headed arrow.*

2001). As an additional control, we injected the anti-*MFmi* morpholino with *noggin* RNA alone, and this has no affect on the expression of the marker genes.

Wnt ligands are at the 'top' end of the wnt canonical signalling pathway, in the sense that they initiate signalling by binding to the *frizzled* receptor (reviewed by Gradl *et al.,* 1999; Yamaguchi, 2001). At the other end of the pathway are the transcription factors that enter the nucleus and affect gene transcription. The most significant of these is the transcription factor *β-catenin* (the vertebrate homologue of the *Drosophila armadillo* gene). Wnt signalling prevents *β-catenin* degradation in the cytoplasm and promotes its entry into the nucleus where, together with another transcription factor, it can mediate changes in the transcription of target genes (reviewed by Gradl *et al.,* 1999; Yamaguchi, 2001). Predictably then, the co-expression of *β-catenin* with *noggin* in caps has a similar affect to that of *Wnt3A* and *noggin*, in that the resulting neural tissue is posteriorised (Domingos *et al.,* 2001; McGrew *et al.,* 1995; Fig. 4B). As *β-catenin* acts downstream of *frizzled* (the binding partner of *Flamingo*), we looked to see if *MFmi* could also block the *β-catenin* induction of the posterior marker genes (i.e. *XHoxb1*, *XHoxb4* and *XHoxb9*). The results of this experiment indicate clearly that it cannot (Fig. 4B), i.e. *MFmi* only blocks *Wnt* mediated posteriorisation, not *β-catenin* mediated posteriorisation.

Flamingo does not affect FGF-Mediated Posteriorisation of the Neural Plate

A number of recent studies have suggested that, in addition to wnt, members of the fibroblast growth factor (FGF) family of secreted proteins are also required for the posteriorisation of the neural plate (Domingos *et al.,* 2001; Ribisi *et al.,* 2000; McGrew *et al.,* 1997). Indeed, it is possible that posteriorisation by wnts is mediated by FGF, as the ectopic expression of an activated form of beta-catenin activates the expression of *FGF3*, *FGF8* and *eFGF* in the *Xenopus* neural plate (Domingos *et al.,* 2001).

In order to exclude the possibility that *Flamingo* interferes with the FGF signalling pathway, rather that than the wnt pathway, we co-expressed *FGF8* with *Wnt3A*, *Noggin* and *Flamingo* in ectoderm (Fig. 5), in exactly the same manner as the experiment described above (Fig. 4A). *FGF8* fully restores the posteriorisation of the neural tissue in this assay, indicating that it is the wnt pathway that *Flamingo* interferes with and not the FGF-dependant step that is probably down stream of wnt.

Flamingo can block the Induction of a Secondary Axis by Wnt Signalling Components

The injection of wnt, or of other components of the canonical wnt pathway into the ventral blastomere of an early *Xenopus* embryo results in the development of a secondary organiser, and subsequently a second body axis complete with a fully formed head (reviewed by Gradl *et al.,* 1999). This therefore represents an additional and independent assay for the canonical wnt pathway. When *Xfz8*, *Xwnt8* or *Xdsh* RNAs were injected into the ventral blastomere of a two cell embryo, a significant proportion of embryos developed with a double axis (Table 1), in agreement with previously published observations (Sokol *et al.,* 1995; Itoh *et al.,* 1998; Smith and Harland, 1991). Injection of *MFmi* RNA in to the dorsal blastomere of two cell embryos did not result in a phenotype (data not shown).

In order to determine whether *Flamingo* can interfere with canonical wnt signalling in this assay, *MFmi* was co-injected with each of these RNAs. This significantly reduces the proportion of embryos with a double axis in the case of *Xwnt8* and *Xfz8* injection, but has no affect on double axis induction by *Xdsh* (Table 1).

Two direct targets of the wnt canonical signalling pathway are the transcription factor *Xsiamois* (*Xsia*; Lemaire *et al.,* 1995; Brannon and Kimelman, 1996; Carnac *et al.,* 1996; Brannon *et al.,* 1997), and the TGF-β - related gene *Xnr3* (Smith *et al.,* 1995;

TABLE 1

mRNA injected (ventral blastomere)	No. of embryos with phenotype: Axis duplication	No. of embryos with phenotype: Normal	No. of dead	No. of specimens	% of specimens with axis duplication
Xwnt8	55	39	5	99	55.6
Xwnt8 + Mfmi	17	61	7	85	20.0
Xfz8	49	10	2	61	80.3
Xfz8 + Mfmi	5	56	8	69	7.2
Xdsh	51	17	8	76	67.1
Xdsh + Mfmi	47	13	9	69	68.1

Flamingo blocks the formation of a double axis induced by *Xwnt8* or *Xfz8* injection into a ventral blastomere. *Xwnt8, Xfz8* or *Xdsh* RNAs, either alone or in combination with *MFmi* RNA, were injected into the ventral balstomeres of an eight cell *Xenopus* embryo. The embryos were then allowed to develop until the late neurula stage, whereupon they were examined for a double axis (an example of which is shown in the table).

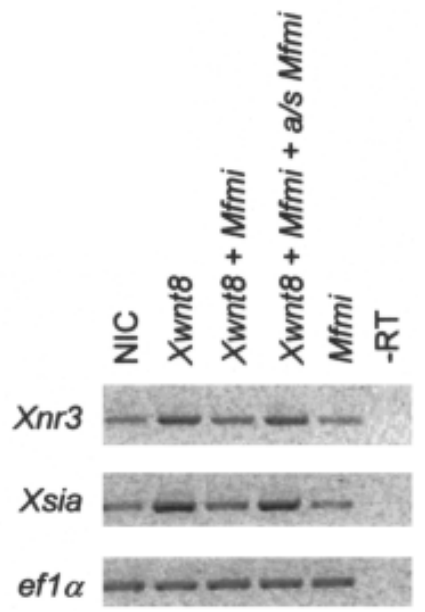

Fig. 6. *MFmi* blocks the up-regulation of *Xnr3* and *Xsia* by *Xwnt8*. *RT-PCR analysis of caps expressing a combination of different injected RNAs, as shown. a/s MFmi, morpholino oligo with complementary sequence to the translation start site of the MFmi RNA.*

McKendry *et al.,* 1997). Both of these are expressed in the organiser of the gastrula stage embryo, and both can induce a secondary axis when injected into the ventral blastomeres. If Flamingo does block the canonical signalling pathway as activated by *Xwnt8*, then the induction of *Xsia* and *Xnr3* should likewise be blocked. To address this, total RNA was extracted from gastrula stage embryos ectopically over-expressing *Xwnt8* and *MFmi*, and the expression of selected genes were assessed by RT-PCR (Fig. 6). Both *Xsia* and *Xnr3* were strongly upregulated by ectopic *Xwnt8* expression, an affect that is negated by the co-expression of *MFmi*, but not *MFmi* with its inhibitory morpholino.

Ectopic XHoxb4 Expression represses XFmi Expression

The data shown in Fig. 4 indicate that *XFmi* can prevent the posteriorisation of anterior neural tissue, thereby blocking the expression of posteriorly expressed *Hox* genes, in particular *XHoxb4* and *XHoxb9*. In order to determine whether this regulatory relationship might by reciprocal, we over expressed *XHoxb4* in fertilised eggs by micro-injecting *XHoxb4* RNA. These eggs were allowed to develop until their untreated siblings had reached the early tail bud stage (26), at which point total RNA was extracted to allow the expression of a number of marker genes to be assessed (Fig. 7). Ectopic expression of *XHoxb4* is known to repress more anteriorly expressed genes, such as *XOtx2* and *XHoxb1* (Hooiveld *et al.,* 1999), whilst upregulating the expression of the endogenous *XHoxb4* gene. Both of these effects are apparent in this experiment (Fig. 7). In addition there is a striking reduction in the number of *XFmi* transcripts detected, indicating that this gene is also down-regulated by *XHoxb4*. As a control, we co-injected the *XHoxb4* RNA with an antisense morpholino oligo that blocks its translation. This prevents the repression of *XOtx2* and *XHoxb1* and *XFmi* expression (Fig. 7).

In agreement with the data presented in Fig. 4, the expression of *MFmi* in embryos significantly reduces the expression of the posteriorly expressed *Hox* genes, *XHoxb4* and *XHoxb9*. When the injected *MFmi* RNA was premixed with an oligo complementary to it translation start site there was no reduction in *Hox* gene expression (Fig. 7).

Discussion

Flamingo in Vertebrates

XFmi is the third vertebrate homologue of *Flamingo* to be cloned, the others genes having been identified in humans and mice. All three share a significant degree of sequence identity at the amino acid level, and the mouse and *Xenopus* clones have very similar expression patterns in early development, especially during gastrulation (Fig. 2; Formstone and Little, 2001). It is therefore likely that the vertebrate *Flamingo* genes have a common function in development, though to date there has been no clear evidence as to what this could be.

Flamingo and the Canonical β-Catenin Signalling Pathway

Flamingo is one of a growing number of genes that have been found to be required for the establishment of tissue polarity in *Drosophila*. Although it is now well established that FLAMINGO protein binds to the FZ receptor, it is still far from clear how this influences the establishment of tissue polarity. FZ itself has a number of well defined functions in development, and one of the most important is acting as a receptor for the *Wnt* ligand in the canonical *Wnt* signaling pathway. This pathway has been subject to extensive study over the last ten years, and is consequently very well characterised. A greatly simplified overview is shown in Fig. 8. Essentially, the *Wnt* ligand binds to the *Fz* receptor resulting in the activation of the phosphoprotein DSH which in turn

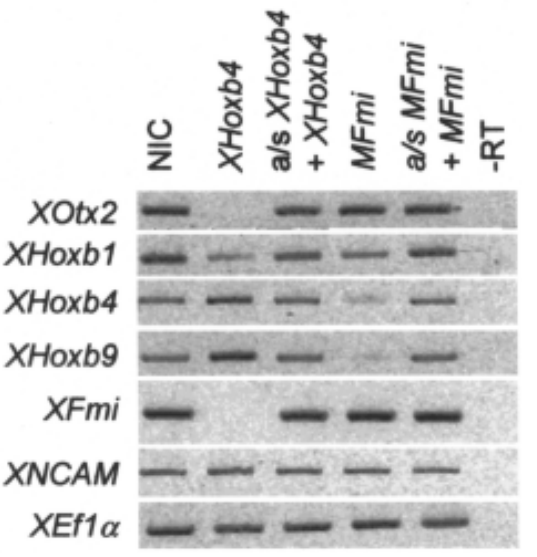

Fig. 7. *Hoxb4* and *Fmi* repress each others expression. *RT-PCR analysis of gene expression in tail bud stage embryos that have developed from eggs injected with different RNAs, as shown above each column. NIC, non-injected control. a/s, co-injection of a morpholino oligo complementary to the translation start site of the injected RNA. -RT, control without reverse transcription. XEf1α is included as a loading control.*

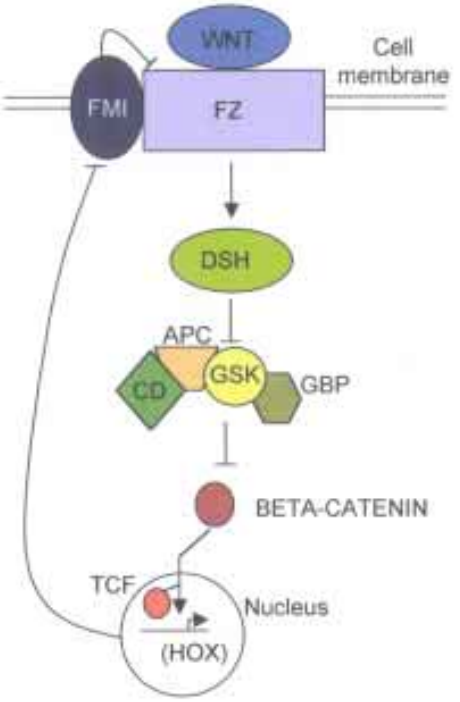

Fig. 8. Putative interactions between *Fmi, Hoxb4* and the canonical wnt signaling pathway. *Fmi interacts with Frizzled and blocks wnt signaling. The wnt pathway activates the expression of Hoxb4 which in turn represses Fmi expression, although it is not clear whether either of these steps are direct. FZ, frizzled; FMI, Flamingo; DSH, dishevelled; APC, adenomatous polyposis coli protein; GSK, glycogen-synthase-kinase-3β; GBP, GSK binding protein; CD, conductin/axin, TCF, T-cell factor.*

inactivates *glycogen-synthase-kinase-3* (*gsk-3β*). This prevents the *gsk-3β* – mediated phosphorylation of *β-catenin* that otherwise targets this protein to the ubiquitin – proteasome pathway. *β-catenin* can then enter the nucleus and, together with its binding partner *Tcf*, can modulate the transcription of its downstream target genes.

In the case of tissue polarity establishment, there is no convincing evidence that any of the wnt signaling pathway is actual involved (apart from *Fz* and *Dsh*), including the *wnt* ligand itself (reviewed by Adler and Lee, 2001). Furthermore, although a number of vertebrate genes with a high level of sequence identity to *Drosophila* tissue polarity genes have been cloned, there is no obvious vertebrate equivalent of the precise tissue polarity examples found in insects (e.g. hair polarity in the wing). The consensus of a number of recent studies on the function of vertebrate tissue polarity homologues suggests that these genes regulate the convergent extension movements that underlie the morphological changes in gastrulation (Park and Moon, 2002; Darken *et al.,* 2002). Interestingly, it is apparent that wnt signaling also regulates these cell movements, although it does so not through the canonical signaling pathway but through components of the JNK kinase cascade (Tada and Smith, 2000; Topczewski *et al.,* 2001; Wallingford *et al.,* 2001; Yamanaka *et al.,* 2002). A third non-canonical wnt pathway, involving a trimeric G-protein and Protein Kinase C (PKC), is required for the correct separation of tissue layers during gastrulation (Winklbauer *et al.,* 2001).

In this study, the ectopic over-expression of *MFmi* is shown to interfere with *wnt* signaling in the anterior – posterior patterning of the neural plate, a process which, like dorsal – ventral patterning, is dependent on the canonical *wnt* pathway. Ectopic *MFmi* expression represses the induction of *XHoxb1*, *XHoxb4* and *XHoxb9* by *XWnt3A*, with the extent of repression increasing in an anterior (*XHoxb1*) to posterior (*XHoxb9*) direction. This may indicate that a higher level of wnt signaling is required to induce the expression of progressively more posterior genes.

As *Fmi* is known to bind to Fz receptors, it would seem likely that *wnt* signaling is blocked at this point (Fig. 8). This is supported by the observation that *β-catenin* mediated posteriorisation of neural tissue is not blocked by ectopic *MFmi*, and that the co-injection of *MFmi* and *XDsh* into the ventral blastomeres of two cell embryos does not block the formation of a secondary axis. These observations raise the possibility then that *Fmi*, whilst not participating in the canonical pathway per se, might be able to influence signaling through it, and hence regulate *wnt*-mediated patterning events. It is of course possible though that *Fmi* has additional, or alternative functions *in vivo*.

Hoxb4 and Fmi repress Each Other's Expression

The repression of one gene's expression by another, and mutual repression between genes are important mechanisms for defining and stabilizing gene expression domains in the early embryo. It is apparent from the data presented here that *XHoxb4* can repress the expression of *XFmi*, and vice-versa (Figs. 7,8). This relationship correlates well with the expression patterns of the two genes, which are essentially complementary (Fig. 2; Godsave *et al.,* 1994).

Although the role of *wnt* / *fz* signaling in controlling *Hox* gene expression is well established in vertebrates (McGrew *et al.,* 1995; Domingos *et al.,* 2001; Yamaguchi *et al.,* 2001), to date there has been little indication that *Hox* genes can regulate *wnt* signaling. The ability of *XHoxb4* to down-regulate *XFmi* expression indicates that this is possible, albeit through an indirect mechanism. It is hoped that future studies will explore the precise nature of these interactions.

Materials and Methods

Constructs

The constructs used for transcribing RNA for micro-injection and the *in vitro* transcription of RNA were as previously described (Hooiveld *et al.,* 1999), with the exception of *MFmi* (accession number AB028499) which was amplified from a cDNA kindly supplied by Dr. T. Uemura and cloned into vector pGEMTeasy. The insert was amplified by PCR, using SP6 and T7 primers, and transcribed using SP6 polymerase. 100 pg of each of the *in vitro* – transcribed RNAs were injected into fertilised eggs, with the exception of *noggin* RNA, of which 300 pg were injected.

RNA Extraction and RT-PCR

Total RNA was extracted from whole embryos using the QuickPrep Total RNA extraction kit (Amersham Pharmacia Biotech Inc.). 3 μg of RNA was used in subsequent reverse transcription reactions. This was mixed with a poly T_{15} oligo to 5 μg/ml and heated to 75°C for 5 minutes. After cooling on ice, the following additional reagents were added; dNTPs to 0.4mM, RNase OUT (Promega) to 1.6 u/μl, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLRvT) RnaseH- point mutant (Promega) to 8u/μl and the appropriate buffer (supplied by the manufacturer) to x1 concentration. The mixture was incubated for one hour at 37°C, heated to 70°C for two minutes and cooled on ice.

PCR reactions were all performed in a total volume of 40 µl. For each we used 1µl of the M-MLRvT reaction (as described above), 0.2 nmols of each primer and 20 µl of Redimix pre-mixed PCR components (Sigma). All reactions were cycled at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds. 30 cycles were used for all primer sets except those for *ef1α*, for which 23 cycles were used. The primers used for *XFmi* amplification were; forward – *XFMIU:* 5' GTG CTT GCC ACT CAG GAT TC 3' and reverse- *XFMID*: 5' GAT TGC CCT TGC AGA GGA T 3'.The sequences of the other primer pairs can be found on the internet at: http://www.sghms.ac.uk/depts/anatomy/index.htm

Embryo Culture and Microinjection

These were performed as described elsewhere (Sive *et al.,* 2000).

Whole Mount* In Situ *Hybridisation

XFmi was cloned into vector pGEMT-easy (Promega), and this was linearised using NcoI. A DIG-labelled *in situ* probe was transcribed from this template using SP6 polymerase. The Otx2 *in situ* probe was made as previously described (Pannese *et al.,* 1995). Probe purification and subsequent *in situ* analysis were performed as described (Sive *et al.,* 2000).

Antisense Oligo Design

An anti-XHoxb4 morpholino oligo was designed, complementary to the translation start site on the mRNA, its sequence being: 5' TGA TCA AAA ACG AAC TCA TTC TCA T 3'. It was synthesised by Gene Tools LLC (USA), and 50pg were injected into fertilised eggs.

Acknowledgement

The authors wish to thank St. George´s Hospital Medical School for their support.

References

ADLER, P. N. and LEE, H. (2001). Frizzled signaling and cell-cell interactions in planar polarity. *Curr. Opin. Cell Biol.* 13: 635-640.

BALAK, K., JACOBSON, M., SUNSHINE, J. and RUTISHAUSER, U. (1987). Neural cell adhesion molecule expression in *Xenopus* embryos. *Dev. Biol.* 119: 540-550.

BLITZ, I. L. and CHO, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene orthodenticle. *Development* 121: 993-1004.

BRANNON, M. and KIMELMAN, D. (1996). Activation of Siamois by the Wnt pathway. *Dev. Biol.* 180: 344-347.

BRANNON, M., GOMPERTS, M., SUMOY, L., MOON, R. T. and KIMELMAN, D. (1997). A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in *Xenopus*. *Genes Dev.* 11: 2359-2370.

CARNAC, G., KODJABACHIAN, L., GURDON, J. B., and LEMAIRE, P. (1996).The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* 122: 3055-3065.

CHAE, J., KIM, M. J., GOO, J. H., COLLIER, S., GUBB, D., CHARLTON, J., ADLER, P.N. and PARK, W. J. (1999). The *Drosophila* tissue polarity gene starry night encodes a member of the protocadherin family. *Development* 126: 5421-5429.

DARKEN, R. S., SCOLA, A. M., RAKEMAN, A. S., DAS, G., MLODZIK, M. and WILSON, P. A. (2002). The planar polarity gene strabismus regulates convergent extension movements in *Xenopus*. *EMBO J.* 21: 976-985.

DOMINGOS, P. M., ITASAKI, N., JONES, C. M., MERCURIO, S., SARGENT, M. G., SMITH, J. C. and KRUMLAUF, R. (2001). The Wnt/beta-catenin pathway posteriorizes neural tissue in *Xenopus* by an indirect mechanism requiring FGF signalling. *Dev. Biol.* 239: 148-160.

FORMSTONE, C. J. and LITTLE, P. F. R. (2001). The *Flamingo*-related mouse Celsr family (Celsr1-3) genes exhibit distinct patterns of expression during embryonic development. *Mech. Dev.* 109: 91-94.

GAO, F. B., BRENMAN, J. E., JAN, L. Y and JAN, Y. N. (1999). Genes regulating dendritic outgrowth, branching, and routing in *Drosophila*. *Genes Dev.* 13: 2549-2561.

GAO, F. B., KOHWI, M., BRENMAN, J. E., JAN, L. Y. and JAN, Y. N. (2000). Control of dendritic field formation in *Drosophila*: the roles of *Flamingo* and competition between homologous neurons. *Neuron* 28: 91-101.

GODSAVE, S., DEKKER, E. J., HOLLING, T., PANNESE, M., BONCINELLI, E. and DURSTON, A. (1994). Expression patterns of Hoxb genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol.* 166: 465-476.

GRADL, D., KÜHL, M. and WEDLICH, D. (1999). Keeping a close eye on Wnt-1/wg signaling in *Xenopus Mech. Dev.* 86: 3-15.

HEASMAN, J., KOFRON, M. and WYLIE, C. (2000). Beta-catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. *Dev. Biol.* 222: 124-134.

HOOIVELD, M. H., MORGAN, R., IN DER RIEDEN, P., HOUTZAGER, E., PANNESE, M., DAMEN, K., BONCINELLI, E. and DURSTON, A. J. (1999). Novel interactions between vertebrate Hox genes. *Int. J. Dev. Biol.* 43: 665-674.

ITOH, K., JACOB, J. and SOKOL S. Y. (1988). A role for *Xenopus* Frizzled 8 in dorsal development. *Mech. Dev.* 74: 145-157.

LAMB, T. M., KNECHT, A. K., SMITH, W. C., STACHEL, S. E., ECONOMIDES, A. N., STAHL, N., YANCOPOLOUS, G. D. and HARLAND, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262: 713-718.

LEMAIRE, P., GARRETT, N. and GURDON, J. B. (1995). Expression cloning of Siamois, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* 81: 85-94.

LU, B., USUI, T., UEMURA, T., JAN, L. and JAN, Y. N. (1999). *Flamingo* controls the planar polarity of sensory bristles and asymmetric division of sensory organ precursors in *Drosophila*. *Curr. Biol.* 9: 1247-1250

MCGREW, L. L., LAI, C. J. and MOON, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* 172: 337-342.

MCGREW, L. L., HOPPLER, S. and MOON, R.T. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*. *Mech. Dev.* 69: 105-114.

MCKENDRY, R., HSU, S. C., HARLAND, R. M. and GROSSCHEDL, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the *Xenopus* nodal-related 3 promoter. *Dev. Biol.* 192: 420-431.

NEWPORT, J. and KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos. *Cell* 30: 675-696.

PANNESE, M., POLO, C., ANDREAZZOLI, M., VIGNALI, R., KABLAR, B., BARSACCHI, G. and BONCINELLI, E. (1995). The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* 121: 707-720.

PARK, M. and MOON, R. T. (2002). The planar cell-polarity gene stbm regulates cell behaviour and cell fate in vertebrate embryos. *Nat. Cell Biol.* 4: 20-25.

RIBISI, S. JR., MARIANI, F. V., AAMAR, E., LAMB, T.M., FRANK, D. and HARLAND, R. M. (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in *Xenopus* laevis. *Dev. Biol.* 227: 183-189.

ROSS, J. J., SHIMMI, O., VILMOS, P., PETRYK, A., KIM, H., GAUDENZ, K., HERMANSON, S., EKKER, S. C., O'CONNOR, M. B. and MARSH, J. L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature* 410: 479-483.

SHIMADA, Y., USUI, T., YANAGAWA, S., TAKEICHI, M. and UEMURA, T. (2001). Asymmetric colocalization of *Flamingo*, a seven-pass transmembrane cadherin, and Dishevelled in planar cell polarization. *Curr. Biol.* 11: 859-863.

SIVE, H. L., GRAINGER, R. M. and HARLAND, R. M. (2000). *Early Development of Xenopus laevis: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

SMITH, W. C. and HARLAND, R. M. (1991). Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* 67: 753-765.

SMITH, W. C., MCKENDRY, R., RIBISI, S. Jr and HARLAND, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* 82: 37-46.

SOKOL, S. Y., KLINGENSMITH, J., PERRIMON, N. and ITOH, K. (1995). Dorsalizing and neuralizing properties of Xdsh, a maternally expressed *Xenopus* homolog of dishevelled. *Development* 121: 1637-1647.

TADA, M. and SMITH, J. C. (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* 127: 2227-2238.

TOPCZEWSKI, J., SEPICH, D. S., MYERS, D. C., WALKER, C., AMORES, A., LELE, Z., HAMMERSCHMIDT, M., POSTLETHWAIT, J. and SOLNICA-KREZEL, L. (2001). The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell* 1: 251-264.

USUI, T., SHIMA, Y., SHIMADA, Y., HIRANO, S., BURGESS, R. W., SCHWARZ, T. L., TAKEICHI, M. and UEMURA, T. (1999). *Flamingo*, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. *Cell*98: 585-595.

WALLINGFORD, J. B., VOGELI, K. M. and HARLAND, R. M. (2001). Regulation of convergent extension in *Xenopus* by Wnt5a and Frizzled-8 is independent of the canonical Wnt pathway. *Int. J. Dev. Biol.* 45: 225-227.

WINKLBAUER, R., MEDINA, A., SWAIN, R. K. and STEINBEISSER, H. (2001). Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. *Nature* 413: 856-860.

YAMAGUCHI, T. P. (2001). Heads or tails: Wnts and anterior-posterior patterning. *Curr. Biol.* 11: R713-R724.

YAMANAKA, H., MORIGUCHI, T., MASUYAMA, N., KUSAKABE, M., HANAFUSA, H., TAKADA, R., TAKADA, S. and NISHIDA, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep.* 3: 69-75.

Received: December 2002
Reviewed by Referees: January 2003
Modified by Authors and Accepted for Publication: February 2003
Edited by: Anne Marie Duprat