Development 127, 1981-1990 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV6428

The putative Wnt receptor *Xenopus* frizzled-7 functions upstream of β-catenin in vertebrate dorsoventral mesoderm patterning

Saulius Sumanas, Peter Strege, Janet Heasman and Stephen C. Ekker*

University of Minnesota Medical School, Department of Genetics, Cell Biology, and Development, Institute of Human Genetics, Room 6-160 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455, USA
*Author for correspondence (e-mail: ekker001@mail.med.umn.edu)

Accepted 10 February; published on WWW 6 April 2000

SUMMARY

We have isolated one member of the frizzled family of wnt receptors from *Xenopus* (Xfz7) to study the role of cell-cell communication in the establishment of the vertebrate axis. We demonstrate that this maternally encoded protein specifically synergizes with wnt proteins in ectopic axis induction. Embryos derived from oocytes depleted of maternal Xfz7 RNA by antisense oligonucleotide injection are deficient in dorsoanterior structures. Xfz7-depleted embryos are deficient in dorsal but not ventral mesoderm due to the reduced expression of the wnt target genes *siamois*, *Xnr3* and *goosecoid*. These signaling defects can be restored by the addition of β-catenin but not Xwnt8b. Xfz7 thus functions upstream of the known GSK-3/axin/β-catenin intracellular signaling complex in vertebrate dorsoventral mesoderm specification.

Key words: *Xenopus*, frizzled, wnt, β-catenin, Axis specification, Dorsal mesoderm

INTRODUCTION

The role of wingless (wnt) protein signaling in establishment of the vertebrate dorsal axis is currently unclear. Misexpression of wnt proteins causes ectopic axes in *Xenopus* embryos (see Moon et al., 1997 for review), suggesting that wnts may be involved in the endogenous dorsal signaling pathway. Furthermore, disruption or depletion of several components of the Xwnt pathway (β-catenin, Heasman et al., 1994; Glycogen-synthase Kinase-3β (GSK-3β), Pierce and Kimelman, 1995; Dominguez et al., 1995; He et al., 1995; axin, Zeng et al., 1997; Xgsk-3-binding protein (GBP), Yost et al., 1998; XTcf3, Molenaar et al., 1996) affect dorsal axis formation. Doubts have been raised recently, however, about the complete conservation of a *Drosophila*-type wingless/pangolin pathway in *Xenopus*. Recent experiments designed to test for this requirement have failed to identify any role for wnt signaling in endogenous dorsal axis specification. For example, a dominant-negative Wnt8 protein (dnXwnt8; Hoppler et al., 1996), a secreted Wnt8-binding protein (Frzb; Wang et al., 1997; Leyns et al., 1997) and a dominant negative Dishevelled protein (Xdd1; Sokol, 1996), each capable of reducing the ability of exogenous wnt proteins to induce ectopic dorsal axes in *Xenopus* embryos, failed to perturb the development of the endogenous axis. Based on these findings, many current research publications (for example, see Glinka et al., 1997; Yost et al., 1998) and reviews (i.e. Wodarz and Nusse, 1998) propose that the dorsal signaling pathway may be activated by a novel intracellular mechanism that does not involve wnts or wnt receptors.

Evidence from *Caenorhabditis elegans*, *Drosophila* and vertebrate studies have demonstrated that the frizzled family of seven-pass transmembrane proteins are essential for wnt-mediated signal transduction in a variety of biological processes (Herman and Horvitz, 1994; Bhanot et al., 1996; Yang-Snyder et al., 1996; He et al., 1997; Slusarski et al., 1997; Harris et al., 1996; Sawa et al., 1996; Thorpe et al., 1997; Rocheleau et al., 1997; Nasevicius et al., 1998; Bhat, 1998; Kennerdell and Carthew, 1998; Muller et al., 1999). This genetic evidence, coupled with prior interaction data (Bhanot et al., 1996) and new biochemical work in *Drosophila* demonstrating direct binding between wnt and frizzled proteins in vivo (Wesley, 1999), argues forcibly for a role as a wnt receptor for this family of serpentine proteins. We have tested whether frizzled gene function is required for axis formation in *Xenopus* to determine the role of cell-cell signaling in this fundamental vertebrate developmental process.

Here we have studied the role of a novel maternal frizzled gene, *Xfz7*, in *Xenopus* axis formation. We report the isolation and characterization of Xfz7. We show that Xfz7 acts synergistically with Xwnt8b, Xwnt11 and Xwnt5a in ectopic axis induction, and that the predicted cytoplasmic terminal residues are essential for this interaction. We study the role of maternal Xfz7 using an antisense depletion approach and provide direct evidence for the requirement of Xfz7 in dorsal axis formation. Depletion of Xfz7 results in loss of dorsoanterior structures and markers of dorsal mesoderm, while partial depletion reduces the ability of ectopic Xwnt8b protein to induce secondary axes. These effects are fully reversible both morphologically and molecularly by the

subsequent introduction of exogenously added Xfz7 transcripts, demonstrating that these antisense-mediated effects are specific for the *Xfz7* gene. We further establish that Xfz7 lies upstream of β-catenin in the dorsal signaling pathway. Thus this work provides direct evidence for a receptor-mediated Xwnt pathway contribution to *Xenopus* axis formation.

MATERIALS AND METHODS

Isolation of *Xenopus* frizzled 7 (Xfz7) cDNA

The degenerate primers YW157 and YW158 (Wang et al., 1996) were used to amplify a 210 bp Xfz7 fragment by PCR from a *Xenopus* oocyte cDNA library (kindly provided by Ernesto Resnik) using TaqHiFi (Boehringer Mannheim) and subsequently ligated into pBluescript (data not shown). This fragment was used to screen a *Xenopus* oocyte λgt10 library (kindly provided by Joe Yost) by colony hybridization (Genius system, Boehringer Mannheim) according to manufacturer's instructions. Three clones were isolated and characterized by direct sequencing. The largest 3 kb Xfz7 cDNA S73 included most of the open reading frame (ORF) and a significant stretch of the 3′UTR. We used library and S73-specific primers to amplify by PCR the 5′ end of Xfz7 ORF. 3′UTR sequences were similarly isolated using standard PCR methods. Both strands of each subclone were subjected to DNA sequence analysis. The composite cDNA sequence has been submitted to GenBank under accession number: AF039215. The complete ORF was isolated by PCR amplification using 5′ and 3′ ORF primers with maternal cDNA as template and confirmed by subsequent sequence analysis. Identification of protein motifs was performed using GeneWorks (2.5).

Evolutionary relationship determination

Full-length protein sequences were aligned using GeneWorks (2.5) evolutionary relationship algorithm. Statistically distinct branchpoints are indicated (data not shown).

Transcript detection

Whole-mount in situ hybridization was performed as described (Harland, 1997). DIG-labeled antisense Xfz7 probe was synthesized using T3 RNA polymerase and DIG RNA labeling kit (Boehringer Mannheim) from cDNA subclone plasmid S73 after linearization with *Bam*HI. Electrophoresis and northern blotting were performed as described (Hopwood et al., 1989). RNA was isolated as described (Gurdon et al., 1985). The 2.7 kb *Eco*RI-*Alw*NI fragment from S73 was used as probe template for Xfz7 detection and labeled with ^{32}P by random priming. In all cases, the ribosomal bands on the transferred membrane were used as loading controls for the relative abundance of RNA loaded in any given lane.

Other constructs used for northern hybridization were Xnr3 (Smith et al., 1995) and Xwnt8 (Christian and Moon, 1993; derived from plasmid XE9 kindly provided by R. Moon). goosecoid (gsc) probe was synthesized by PCR amplification of gsc ORF fragment from gsc

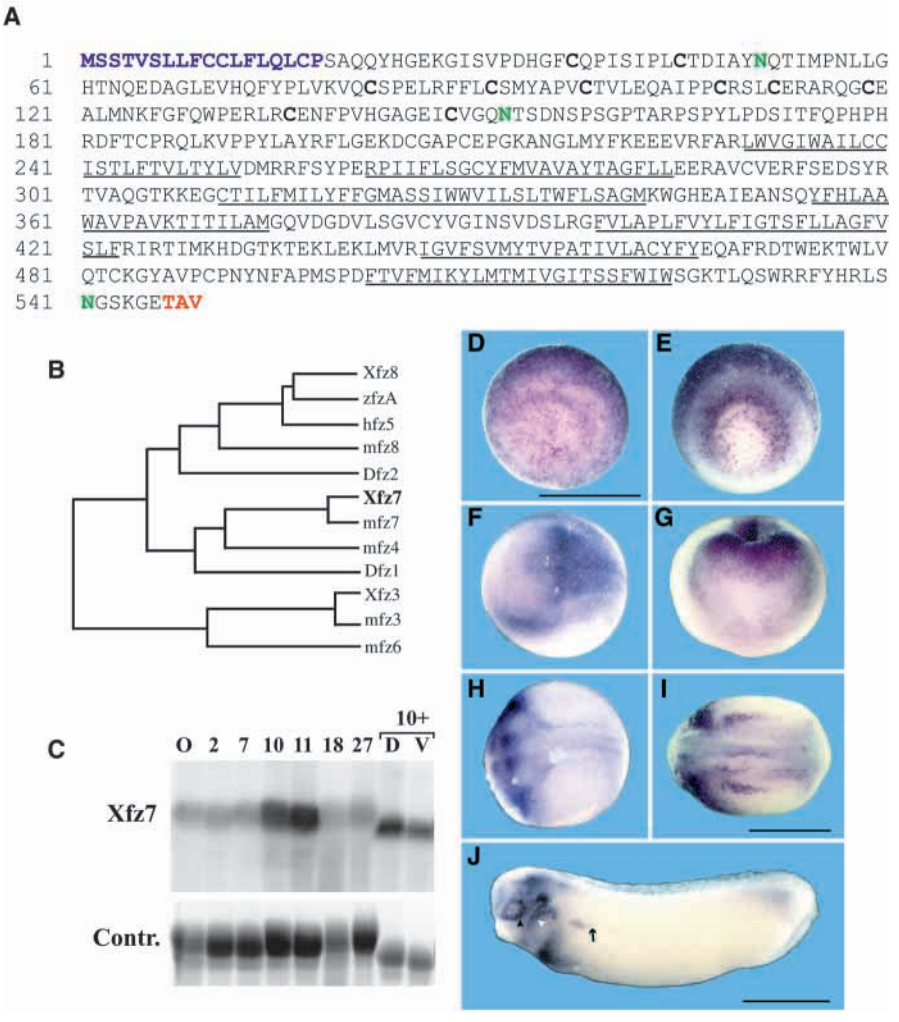

Fig. 1. *Xenopus laevis* frizzled 7 (Xfz7) sequence and expression in embryonic development. (A) Deduced Xfz7 protein sequence. The amino-terminal signal sequence is shown in purple, conserved cysteines are in bold, and the putative PDZ-binding motif is in red. Consensus N-linked glycosylation sites are in green, and the putative transmembrane domains are underlined. (B) Estimated evolutionary relationship of Xfz7 with other known frizzled proteins. Proteins displaying the greatest sequence similarity cluster together. Using this analysis, three highly distinctive structural groups were identified. (C) Northern analysis of Xfz7 expression. O, 2, 7, 10, 11, 18, 27 denote oocyte, and stages 2-27, respectively. D, V lanes contain RNA from dorsal and ventral halves of stage 10+ embryos. Only a single 4.6 kb band is observed in all stages examined. (D,E,G,J) dorsal is up, (F,H-J) anterior is to the left. (D-J) Xfz7 distribution as determined by in situ hybridization. The scale bars correspond to 1 mm. (D,E) Animal and vegetal views of stage 11 embryo. Note the preferential dorsal enrichment of Xfz7 transcripts at this stage of development that correlates with the northern data. (F,G) Dorsal and rostral views of stage 14 embryo. Staining is apparent at, but not limited to, anterior neural folds. (H) Dorsal view of stage 17 embryo. Expression is apparent at neural folds and more weakly in the somites. (I) Dorsal view of stage 20 embryo. Xfz7 RNA is found in the neural folds and forming pharyngeal arches. (J) Lateral view of stage 27 embryo. Black arrowhead marks the eye, white arrowhead labels the pharyngeal arches, and black arrow is the pronephros.

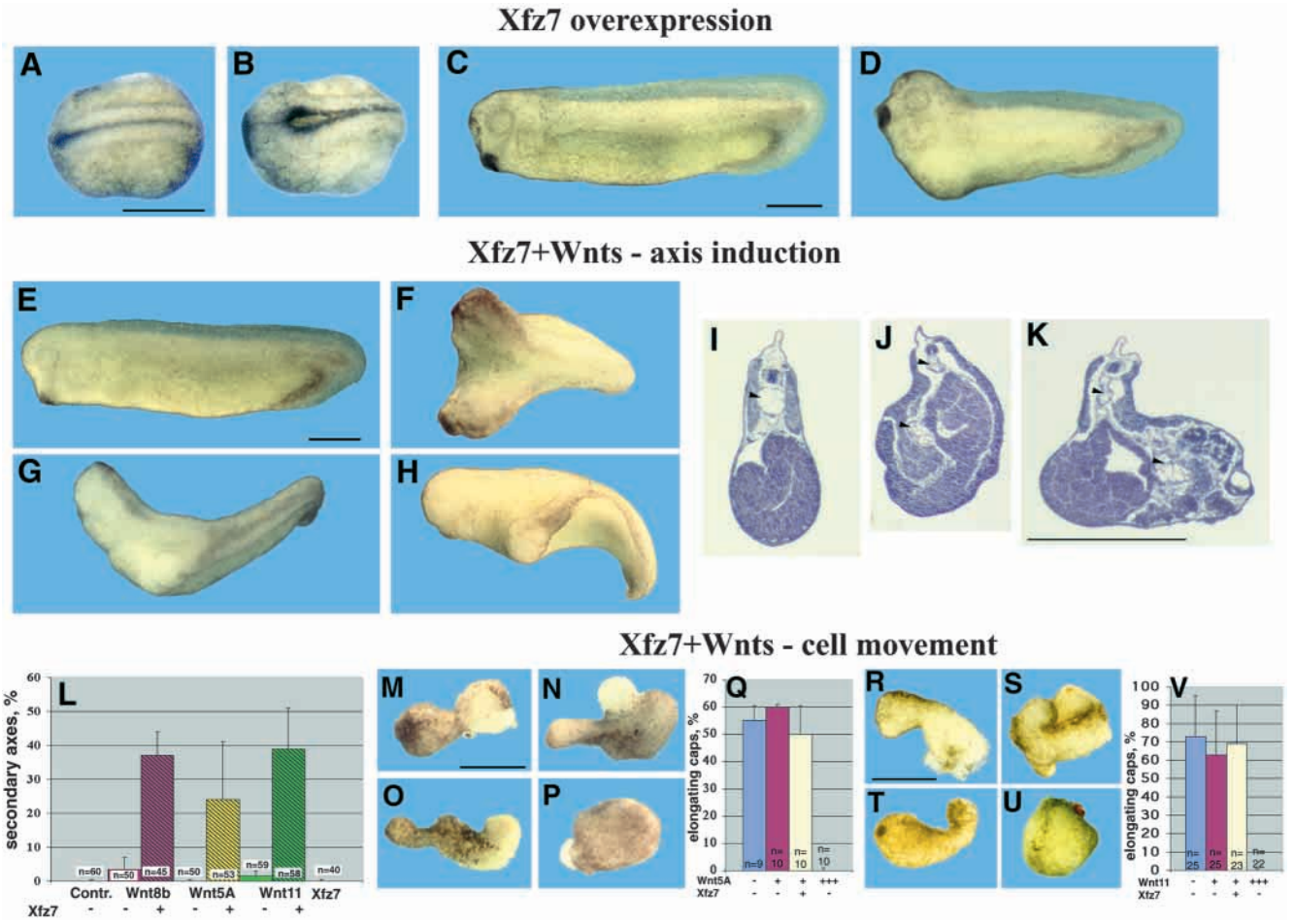

Fig. 2. Xfz7 synergizes with Wnts in axis induction. The scale bars correspond to 1 mm. All embryos shown are at tailbud stages except as noted. (A,C) Control uninjected embryo. (A,B) Overexpression of Xfz7 interferes with neural fold closure at neurula stage. (C,D) Overexpression of Xfz7 results in shortened, bent embryos. (E-H) Coinjection of Xfz7 with either Wnt8b, Wnt5A or Wnt11 results in axis duplication. (E) Control uninjected embryo. (F) Wnt8b+Xfz7-injected embryo. (G) Wnt5A+Xfz7-injected embryo. (H) Wnt11+Xfz7-injected embryo. (I-K) Secondary axes of Wnt5A+Xfz7 and Wnt11+Xfz7-injected embryos contain notochords, marked by arrowheads. (I) Control uninjected embryo. (J) Wnt5A+Xfz7-injected embryo. (K) Wnt11+Xfz7-injected embryo. (L) Xfz7 synergizes with either Wnt8b, Wnt5A or Wnt11 in the induction of a secondary axis. (M-Q) Coinjection of Wnt5A and Xfz7 does not block activin-induced elongation of animal caps. (M) Control uninjected activin treated animal cap, (N) 1× dose Wnt5A-injected animal cap. (O) 1× dose Wnt5A+Xfz7-injected animal cap. (P) 3× dose Wnt5A-injected animal cap. Note that 3× Wnt5A injection blocks elongation while 1× Wnt5A+Xfz7 does not. (Q) Xfz7 does not synergize with Wnt5A in blocking the elongation of activin-treated animal caps. (R-V) Coinjection of Wnt11 and Xfz7 does not block activin-induced elongation of animal caps. (R) shows a control uninjected activin treated animal cap, (S) shows 1× Wnt11-injected animal cap, (T) shows 1× Wnt11+Xfz7-injected animal cap, (U) shows 3× Wnt11-injected animal cap. Note that 3× Wnt11 injection blocks elongation while 1× Wnt11+Xfz7 does not. (V) Xfz7 does not synergize with Wnt11 in blocking the elongation of activin-treated animal caps.

plasmid (Christian and Moon, 1993, generous gift of R. Moon) and subsequent ^{32}P labeling of this PCR fragment.

RT-PCR for siamois and histone H4 was performed as described (Zhang et al., 1998) using one embryo for each reaction. The primers and PCR profile for siamois and histone H4 were essentially as described (Darras et al., 1997, and Pillemer et al., 1998, respectively), except that 26 amplification cycles for siamois were used. To detect Xfz7 and Xfz3, we used non-radioactive PCR with the following cycle profile: 94°C denaturation for 30 seconds, 57°C annealing for 30 seconds, 72°C amplification for 15 seconds. The following primer pairs were used (5′ to 3′): Xfz7 (forward – TGCCGGCCAC-CATCGTGCTG, reverse – CCTGCGCCAGGACTGTAGGGT; 26 cycles); Xfz3 (forward – TGGCGTGCCGTGGCCGGAAG, reverse – GTGGCAGAGAGGCAAACTATGG; 30 cycles). In both cases, amplification was found to be in the linear range (data not shown).

Xfz7 injection constructs

Plasmid Xfz7/T3TS was constructed by the PCR amplification of the Xfz7 ORF from a maternal cDNA library and subsequently subcloned into pT3TS (Hyatt and Ekker, 1999). Xfz7ΔT/T3TS was generated by PCR using Xfz7/T3TS as template and subsequently subcloned into pT3TS. Each construct was confirmed by DNA sequence analysis.

RNA synthesis

All synthetic RNA used in this study was made by in vitro transcription using mMessage mMachine kit (Ambion) according to manufacturer's instructions. RNA concentration was estimated from the intensity of ethidium bromide staining in the agarose gel.

*Xba*I-linearized Xfz7/T3TS or Xfz7ΔT/T3TS DNA was used as template for RNA synthesis. We used the following templates for wnt RNA synthesis: Xwnt8b in pCS2+ (Cui et al., 1995), XWnt11 in

pSP64T (Ku and Melton, 1993), and XWnt5A in pSP64T (Moon et al., 1993; gift of R. Moon).

Xfz7 and Xfz7ΔT misexpression

For injections of Xfz7 and Xfz7ΔT, dejellied embryos at the 2-cell stage were placed in 3% Ficoll in 1× MMR and injected into both cells. 2 ng total of Xfz7 or Xfz7ΔT RNA were injected in the overexpression experiments. Embryos were placed into 0.1× MMR at the midblastula stage for further development. For injections of Xfz7ΔT RNA into oocytes, 2.5 ng of RNA was injected into marginal zones of defolliculated oocytes 4 hours prior to progesterone stimulation. Oocytes subsequently were fertilized using host-transfer technique as described below.

Co-injection of Fz and Wnts

3-10 pg Wnt8b, 600-900 pg Wnt11 or 300 pg Wnt5A were injected into embryos alone or in conjunction with 0.5-1 ng of Xfz7 (or Xfz7ΔT) into one ventral blastomere at the 4-cell stage. The injected embryos were scored for double axes at tailbud stages. For cell movement assays, embryos were injected into both blastomeres at the 2-cell stage with 200 ng of Wnt5A or 200-600 pg of Wnt11 with or without 0.5 ng of Xfz7. Animal caps from the experimental and control embryos were dissected at the midblastula stage and placed into 1× MMR containing 3 ng/ml bovine activin A (Genzyme) and cultured until sibling stage 15.

For histological analysis, sample embryos were fixed in MEMFA at stages 28-32, embedded in paraplast, sectioned into 20 μm sections and stained with hematoxylin as described (Kelly et al., 1991).

Depletion of maternal Xfz7

For Xfz7 depletion experiments, ovaries were isolated and oocytes manually defolliculated, as described previously (Heasman et al., 1994). The oocytes were injected into the equatorial region with a combination of 2 or 3 ng of oligo Xfz7-37S (sequence: C*T*G*TTCCAACACGGT*G*C*A, asterisk represents phosphothioate linkages) and 2 or 3 ng of oligo Xfz7-43S (sequence: G*T*T*CATGAGCGCCTC*G*C*A) HPLC-purified antisense oligonucleotides (Genosys). The oocytes were cultured for 24 hours, matured for 10 hours following stimulation with 2 μM progesterone, labeled using vital dyes and fertilized using the host transfer technique, as described previously (Heasman et al., 1994). Sample embryos were frozen at stage 10+ as defined by the appearance of the dorsal lip, for marker analysis. In three independent experiments where oocytes were injected with 5.5-6 ng of the oligo mixture, at tailbud stages 22 embryos total were normal (DAI=5), 22 were categorized as DAI=3-4, 26 corresponded to DAI=1-2, and 6 were evaluated as DAI<1. Average DAI = 2.9±0.4. Corresponding numbers of uninjected control embryos were 74, 7, 4 and 0, respectively (DAI = 4.7±0.3).

Histological analysis was performed on stage 40 embryos as described above.

Xnr3 expression was reduced in 14 out of 17 Xfz7-depleted embryo samples (2 embryos per sample) analyzed in 7 independent experiments. Representative membranes from the Xnr3 analyses were reprobed for *goosecoid* and *wnt8* gene expression (Fig. 5A, lanes 1-4). Reduction in *siamois* level was observed in 9 out of 13 single Xfz7-depleted embryos analyzed in 3 independent experiments.

Individual analyses of Xfz7-depleted embryos (stage 10+) noted extreme examples of Xnr3 reduction (Fig. 5A, lanes 5 and 6). This level of reduction occurred in 8 out of 30 embryos analyzed in 4 independent experiments.

To compare the depletion level of Xfz7 in oocytes injected with different doses of oligonucleotide, northern blots were exposed to a PhosphorImager screen, and the amount of RNA was quantified using the IPLabGel program.

To analyze specificity of the depletion phenotype, 300 pg of synthetic Xfz7 RNA was injected into marginal zones of Xfz7-depleted oocytes 4 hours prior to progesterone stimulation. For epistasis analysis, 4-10 pg of Wnt8b or 200-450 pg of β-catenin RNA was injected into marginal zones of Xfz7-depleted oocytes 4 hours prior to progesterone stimulation.

Wnt interaction specificity studies

To test the specificity of Wnt response in Xfz7-depleted embryos, 10-20 pg of Wnt8b RNA was injected into one of the ventral cells at the 4-cell stage into control and depleted embryos. The injected embryos were scored for double axes at tailbud stages. 300 pg of Wnt5A or 600 pg of Wnt11 RNA was injected at the 2-cell stage into both blastomeres of control and Xfz7-depleted embryos. Animal caps from these embryos were dissected at the midblastula stage, placed into 1× MMR containing 3 ng/ml bovine activin A (Genzyme) and cultured until sibling stage 15.

RESULTS

Isolation of the putative wnt receptor *Xenopus* frizzled 7 (Xfz7)

We isolated and characterized a member of the *Xenopus* frizzled gene family of wnt receptors. The transcribed RNA is predicted to encode a 549 amino acid (a.a.) residues protein with significant homology to the fz family of serpentine receptors (Fig. 1A). BLAST search of nucleotide and protein databases using the sequence of the isolated *Xenopus* frizzled homologue identified an exceptionally high homology to mouse frizzled 7 (88% identical a.a. residues), therefore it was called *Xenopus* frizzled 7 (Xfz7). Xfz7 contains features diagnostic of other frizzled family members, such as an amino-terminal signal sequence (Fig. 1A, purple residues), an amino-terminal cysteine-rich domain (Fig. 1A, conserved cysteines in bold), and seven predicted membrane spanning domains (Fig. 1A, underlined). A relatively short poorly conserved cytoplasmic tail with a single PDZ domain binding motif (S/T-X-V; Fig. 1A, red residues) was also noted. Xfz7 falls into the Dfz1 class of fz proteins (Fig. 1B), with 50% identical a.a. residues to Dfz1.

The expression of Xfz7 was characterized using northern analysis and whole-mount in situ hybridization (Fig. 1C-J). Maternal and zygotic Xfz7 transcripts were identified (Fig. 1C). Early maternal transcripts were detected in both animal and vegetal hemispheres, as analyzed by northern hybridization (data not shown). At the late blastula-gastrula stages, Xfz7 is concentrated dorsally and partially excluded from the yolk plug (Fig. 1C-E). During neurulation Xfz7 expression becomes localized to neural folds in presumptive neural-crest-cell-derived areas (Fig. 1F-H). In the late neurula, Xfz7 is localized to some neural crest cell derivatives such as pharyngeal arches and somites (Fig. 1I). By the tailbud stage, Xfz7 transcripts are detected in the eye, ear, heart regions, pharyngeal arches and pronephros (Fig. 1J).

Overexpression of Xfz7 to test role of Xfz7 in early development

Up to 2 ng of Xfz7-encoding mRNA was microinjected into both cells of 2-cell-stage *Xenopus* embryos and assayed at subsequent stages of development. At the neurula stage, Xfz7 RNA-injected embryos display incompletely closed neural folds (Fig. 2A,B), which is similar to the results for *Xfz3* (Shi et al., 1998) or zebrafish frizzled genes (S. S. and S. C. E.,

unpublished data). At tailbud stages, Xfz7-injected embryos display shortened axes and are bent dorsally (Fig. 2C,D). In contrast to Xfz8 (Itoh et al., 1995; Deardorff et al., 1998), ventral blastomere injection of Xfz7 does not result in axis duplication (Fig. 2L, last column). In zebrafish embryos, Xfz7 was capable of dorsalization and axis induction (data not shown) as was observed for zebrafish frizzled A and human frizzled 5 genes (Nasevicius et al., 1998).

Xfz7 synergizes with Wnts in secondary axis induction

To identify candidate ligands for Xfz7, we tested the ability of Xfz7 to synergize with the known maternally expressed Wnt proteins, Wnt5A, Wnt8b and Wnt11, in co-injection experiments. Of these three Wnts, only Wnt8b and Wnt11 are known to induce secondary axes when expressed ectopically. We co-injected Xfz7 with Wnts into ventral blastomeres at doses where Wnts alone produced a very low percentage of axis duplications. Co-injection of Xfz7 with Wnt8b, Wnt5A or Wnt11 greatly increased the number of observed ectopic axes (Fig. 2E-H,L). Secondary notochords were observed in a number of sectioned Wnt11+Xfz7 and Wnt5A+Xfz7-injected embryos (Fig. 2I-K). This synergy provides evidence that Xfz7 is likely to function as a wnt receptor during development.

Xfz7 does not synergize with Wnts in affecting morphogenetic movements

Wnt proteins fall into two distinct functional classes. Wnt-1 class can cause axis duplication when expressed ectopically, which is thought to occur through β-catenin-dependent mechanism (review by Gradl et al., 1999). Wnt5A and Wnt11 class does not cause a significant percentage of ectopic axes but are known to block the elongation of activin-treated animal caps, which mimics the morphogenetic movements normally associated with gastrulation (Moon et al., 1993; Du et al., 1995), an effect believed to be β-catenin independent (Gradl et al., 1999). If Xfz7 was acting as a receptor in this pathway, we would expect Xfz7 to enhance the ability of cells to respond to limiting amounts of wnt proteins. We co-injected Xfz7 with Wnt5A or Wnt11 into both cells at the 2-cell stages at doses where Wnts produce little or no inhibitory effect on the elongation of animal caps (uninjected animal caps, Fig. 2M,R; animal caps injected with low doses of Wnt5A or Wnt11, Fig. 2N and Fig. 2S, respectively). Co-injection of Xfz7 with either Wnt5A or Wnt11 had no significant effect on the elongation of animal caps (see Fig. 2O,Q for Wnt5A+Xfz7, and Fig. 2T,V for Wnt11+Xfz7). Internal controls for Xfz7 and Wnt activities were included in these experiments, e.g., high doses of Wnt5A or Wnt11 RNA which could inhibit animal cap elongation (Fig. 2P and 2U, respectively). Thus Xfz7 does not synergize with Wnt5A or Wnt 11 in inhibiting morphogenetic movements, and Xfz7 is not likely to be functioning as a receptor in this wnt-mediated bioassay.

Moderate depletion of maternal Xfz7 transcripts blocks signaling by axis-inducing Wnts

To address maternal function of Xfz7, we undertook a depletion by antisense oligonucleotides approach. We identified Xfz7-specific antisense oligonucleotides (see Materials and Methods) for which an injected dose of 4 ng per oocyte resulted in the significant depletion of endogenous Xfz7 transcripts (to 12±6% of endogenous levels), as analyzed by northern hybridization (Fig. 3G). These moderate-level Xfz7-depleted embryos produced no detectable phenotype, however, as analyzed morphologically or by early mesoderm markers (Fig. 3B,E; data not shown). To test if Wnt signaling is affected in Xfz7-depleted embryos and to develop a functional assay for these knock-down studies, we injected maternally expressed Wnt8b into a ventral blastomere of Xfz7-depleted embryos. The number of ectopic axes was significantly lower in Xfz7-depleted embryos compared to control embryos injected with Wnt8b alone (Fig. 3A,C,F). Injection of synthetic Xfz7 RNA into Xfz7-depleted oocytes restored the ability of ectopic Wnt8b to induce secondary axes (Fig. 3D,F), demonstrating the specificity of the antisense depletion to Xfz7. A partial reduction in Xfz7 levels is thus sufficient to compromise the ability of Wnt8b to induce ectopic axes without altering the establishment of endogenous dorsoventral mesoderm.

To test the ability of Wnts to inhibit morphogenetic movements in Xfz7-depleted embryos, we injected Wnt5A and Wnt11 into Xfz7-depleted embryos. Animal caps were explanted, treated with activin and scored for elongation. Depletion of Xfz7 did not significantly affect the ability of Wnt5A or Wnt11 to block elongation (Fig. 3H-M). Thus Wnt signaling leading to the inhibition of morphogenetic movements is not affected in embryos moderately depleted of maternal *Xfz7* gene function. We cannot exclude a formal possibility though that the blocking of Wnt inhibition is rescued by the zygotic Xfz7 since its transcription starts soon after the animal caps are explanted.

Strong Xfz7 depletion results in ventralized embryos

To test if Xfz7 has any endogenous role, we optimized the depletion of maternal Xfz7 transcripts by increasing the amount of oligonucleotide mixture injected into oocytes (up to 6 ng). This dose of Xfz7 antisense oligonucleotides resulted in the nearly quantitative depletion of endogenous maternal Xfz7 transcripts (to 2.0±1.9% of endogenous levels), as assayed using northern analyses (Fig. 4F). 70% of the resulting experimental embryos had various degrees of deficiencies of dorsoanterior structures (Fig. 4A-D,J). The dorsoanterior index (DAI; Scharf and Gerhart, 1983; Kao and Elinson, 1988) was used to estimate the degree of ventralization more precisely at late tailbud stages. Phenotypes ranged from mild reduction of anterior head structures (Fig. 4B) to almost complete loss of dorsal mesodermal structures (Fig. 4D). Histological analysis of the most extremely ventralized embryos revealed the absence of any dorsal structures (Fig. 4H,I). These embryos were reminiscent of those deprived of dorsal mesoderm resulting from UV-irradiation (Scharf and Gerhart, 1983).

Specificity of Xfz7 depletion phenotype

If the observed phenotype is due to the loss of Xfz7 function, injection of synthetic RNA into depleted oocytes would be expected to rescue the phenotype. Indeed, injection of synthetic Xfz7 RNA into Xfz7-depleted oocytes significantly reduced ventralization, as judged by the significantly higher percentage of normal embryos and the change of distribution of any affected embryos to weaker DAI categories upon the re-introduction of Xfz7 message (Fig. 4E,J). Fig. 4F demonstrates that we have effectively replaced endogenous Xfz7 transcripts with exogenous mRNA in these RNA rescue experiments.

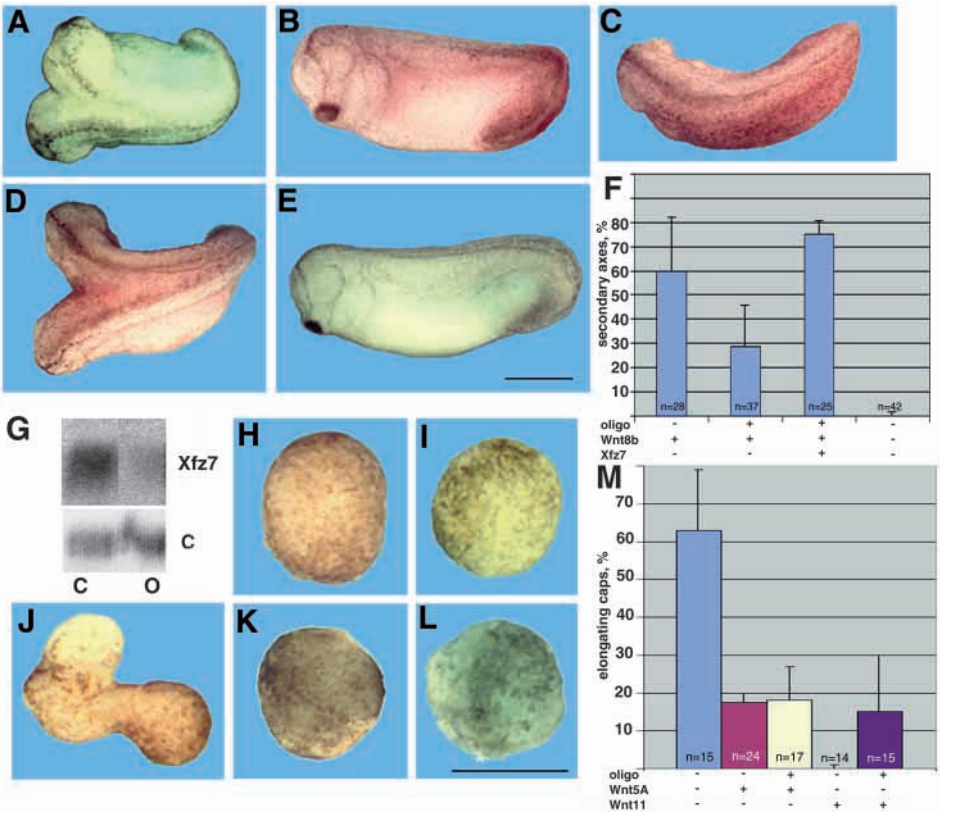

Fig. 3. Moderate depletion of Xfz7 blocks wnt induced axis duplication. (A-F) Injection of Wnt8b into Xfz7-depleted embryos results in a significantly lower percentage of secondary axes. 4 ng total Xfz7 antisense oligonucleotides (oligo) was used in these experiments, which resulted in a moderate depletion (to 12%±6% endogenous transcript levels) of maternal Xfz7 transcript (see Panel G). The scale bar corresponds to 1 mm. All embryos shown are at tailbud stages. (A) Wnt8b-injected embryo with ectopic axis. (B) Xfz7-depleted embryo derived from oocytes injected with oligo alone. (C) Wnt8b-injected, Xfz7-depleted embryo. (D) Xfz7-depleted embryo injected with Wnt8b and Xfz7. (E) Control uninjected embryo. (G) A northern blot, probed for Xfz7, of RNA from oocytes after injection with 4 ng of antisense oligonucleotide mixture. C and O represent uninjected control and oligo-injected oocytes, respectively. (H-M) Depletion of Xfz7 does not alleviate Wnt5A and Wnt11 inhibition of activin-induced elongation of animal caps. 4 ng of oligonucleotide mixture was used in these experiments. Scale bar corresponds to 1 mm. (H) Activin-treated animal cap from Wnt5A-alone-injected embryo. (I) Animal cap from Xfz7-depleted and Wnt5A-injected embryo. (J) Animal cap from an uninjected control embryo. (K) Animal cap from Wnt11-injected embryo. (L) Animal cap from Xfz7-depleted and Wnt11-injected embryo.

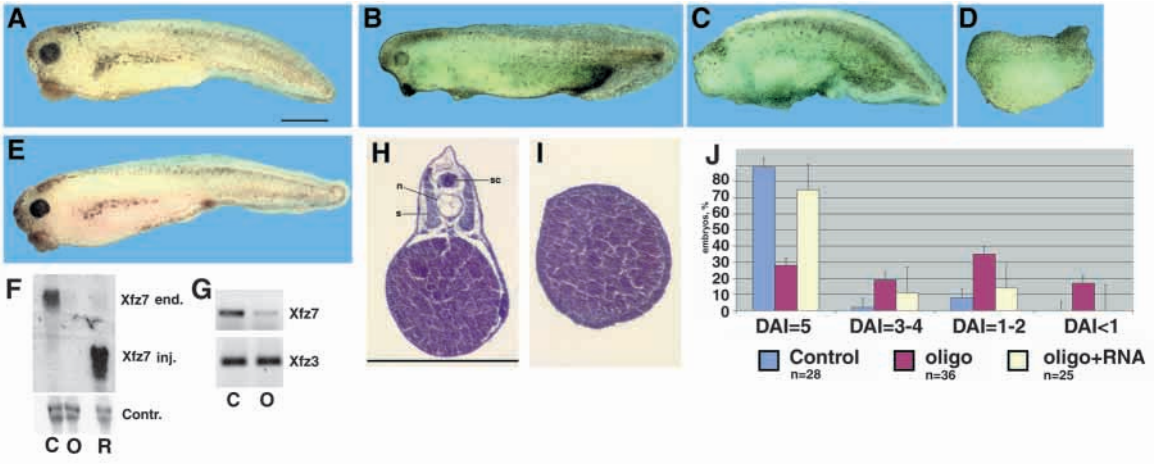

Fig. 4. Formation of dorsal but not ventral mesoderm requires Xfz7 function. 5.5-6 ng of Xfz7 antisense oligonucleotide mixture was used in these experiments which resulted in a strong depletion of maternal Xfz7 transcript (to 2.0%±1.9% of endogenous Xfz7 levels; see F). The scale bars correspond to 1 mm. Embryos shown are at late tailbud stages. (A-E,H-J) Morphological analysis of the Xfz7 depletion phenotype. Depletion of maternal Xfz7 reduces dorsoanterior structures to different extent. These phenotypes are rescuable by injecting back Xfz7 RNA into depleted oocytes. (A) Uninjected control embryo. (B-D) Various degrees of ventralization are observed in embryos strongly depleted of Xfz7 transcripts. (B) Representative embryo from DAI=3-4 group. (C) Representative embryo from DAI=1-2 group. (D) Representive embryo from DAI<1 group. (E) Embryo that was injected with 6 ng of Xfz7 antisense oligonucleotide mixture and with 300 pg of Xfz7 RNA. (F) A northern blot, probed for Xfz7, of RNA from oocytes, injected with 6 ng of antisense oligonucleotide mixture. C, O and R represent uninjected control, oligo- and oligo+Xfz7 RNA-injected oocytes, respectively. Note that synthetic Xfz7 RNA is shorter than endogenous Xfz7 RNA. (G) RT-PCR analysis of the two known maternally expressed *Xenopus* frizzled homologs Xfz7 and Xfz3 in control (C) and oligonucleotide-injected (O) oocytes. Note that the oligo mixture specifically depleted Xfz7 but not Xfz3. (H,I) Transverse sections of the extremely ventralized Xfz7-depleted and control embryos. Note the absence of any dorsal structures in the extreme Xfz7-depleted embryos. (H) Control uninjected embryo. Spinal cord (sc), notochord (n), and somites (s) are marked. (I) Xfz7-depleted embryo.

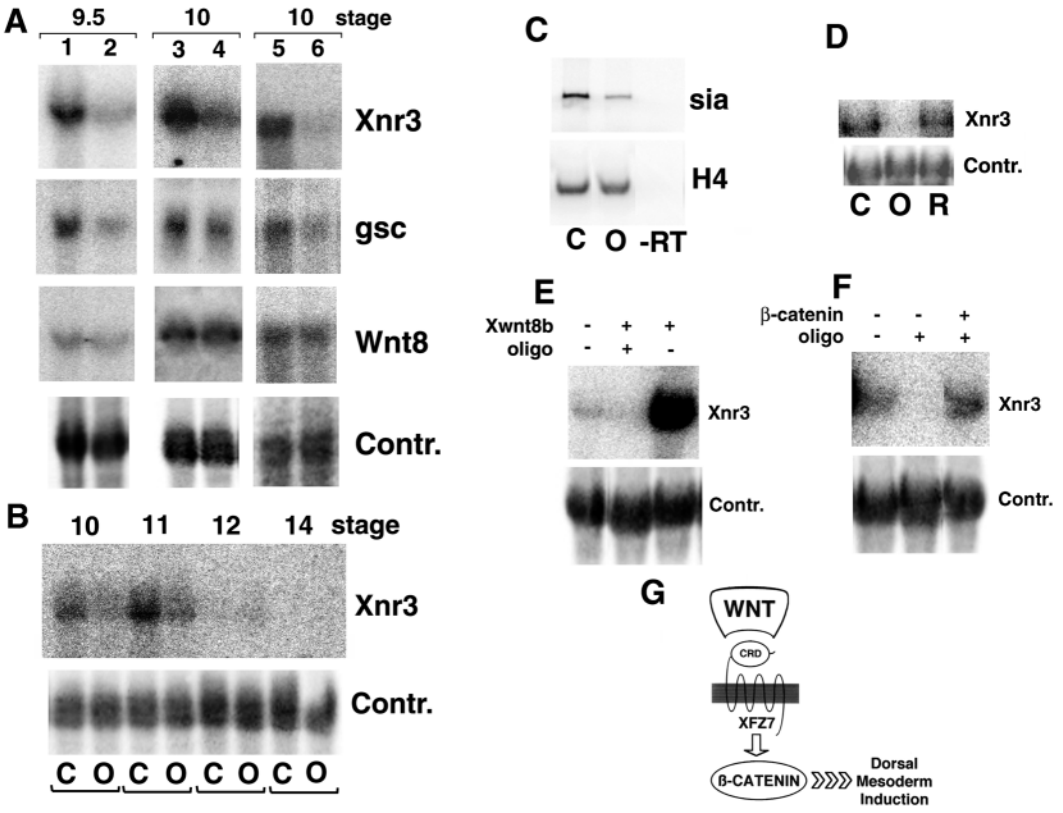

Fig. 5. Xfz7 functions downstream of Wnt and upstream of β-catenin in the dorsal mesoderm induction pathway. (A-D) Molecular analysis of Xfz7 depletion phenotype. (A) Northern analysis of different markers in uninjected controls and oligonucleotide-injected embryos. Stage 9.5 representative control and oligo-injected embryos, lanes 1, 2 (2 embryos per lane); stage 10+ representative control and oligo-injected embryos, lanes 3, 4 (2 embryos per lane); stage 10+ extreme control and oligo-injected embryos, lanes 5, 6 (1 embryo per lane). Note the reduction in the expression of dorsal mesoderm markers *Xenopus* nodal related-3 (Xnr3) and goosecoid (gsc) in the Xfz7-depleted embryos and absence of such reduction in the ventral mesoderm marker Wnt8. (B) Northern analysis of Xnr3 expression at gastrula and early neurula stages in uninjected control (C) and oligonucleotide-injected (O) embryos. Note that Xnr3 expression in Xfz7-depleted embryos is reduced and never reaches control embryo levels. (C) RT-PCR analysis of the Wnt target gene *siamois* (*sia*) expression in control (C), and oligo-injected (O) stage 10+ embryos. Note marked reduction in its expression in oligo-injected embryos. Histone H4 expression was used as a control for cDNA amount in RT-PCR reaction. (D) Injection of synthetic Xfz7 RNA into Xfz7-depleted oocytes restores the expression of a dorsal mesoderm marker Xnr3, as analyzed by northern blotting. C, O and R represent uninjected control, oligo- and oligo+Xfz7 RNA-injected oocytes, respectively. (E) Wnt8b induces dorsal mesoderm marker Xnr3 in uninjected control embryos but not in Xfz7-depleted embryos, as analyzed by northern blotting. (F) β-catenin induces Xnr3 in Xfz7-depleted embryos, as analyzed by northern blotting. (G) A model depicting Xfz7 role in the dorsal mesoderm induction pathway.

Each of the oligonucleotides when injected individually at doses up to 5 ng did not cause any significant phenotype (data not shown). The combination of the two oligonucleotides at 3 ng each resulted in more efficient depletion than each of them separately at 6 ng. It is highly unlikely that both oligonucleotides would by coincidence target the same RNA.

To further demonstrate that the ventralization occurs due to specific loss of Xfz7 RNA and not any other frizzled RNA, we analyzed the expression of the only other known maternally expressed Xfz3 homolog (Shi et al., 1998). Xfz3 expression in oligo-injected oocytes was not affected, as analyzed by RT-PCR (Fig. 4G).

Strong Xfz7 depletion specifically reduces the expression of dorsal mesodermal markers

Sample embryos were frozen before gastrulation (stage 9.5) and at the start of gastrulation (stage 10+, as determined by appearance of dorsal lip in each embryo being analyzed) and analyzed by northern hybridization for expression of dorsal and ventral mesoderm markers. Representative samples (2 embryo equivalents per lane) are shown in Fig. 5A, lanes 1-4; the more extreme cases presumably corresponding to DAI<1 embryos are represented in Fig. 5A, lanes 5-6 (1 embryo per lane). Expression of the dorsal marker Xnr3, a known direct target of β-catenin signaling (Smith et al., 1995; McKendry et al., 1997), was significantly reduced in Xfz7-depleted embryos (Fig. 5A; n=7 experiments, see Materials and Methods). Expression of another direct target of Wnt signaling, *siamois* (Fan et al., 1998; Brannon et al., 1997; Lemaire et al., 1995), was reduced in Xfz7-depleted embryos, as analyzed by RT-PCR (Fig. 5C). Another known dorsal mesoderm marker goosecoid (gsc; Cho et al., 1991) was reduced while expression of a ventral mesodermal marker Wnt8 (Christian et al., 1991) was not affected (Fig. 5A). A later stage analysis demonstrates that Xnr3 levels in Xfz7-depleted embryos never reach control embryo levels (Fig. 5B), indicating that these defects in dorsal marker induction are not due to a simple delay in embryonic development. Injection of synthetic Xfz7 RNA into Xfz7-depleted oocytes restored *Xnr3* expression (Fig. 5D). These results show that *Xfz7* gene function is necessary for the induction of dorsal mesoderm.

Xfz7 functions between Wnt and β-catenin in dorsal mesoderm induction

To test whether Xfz7 functions in the known early Wnt pathway, we injected Xwnt8b into Xfz7-depleted oocytes (6 ng of oligonucleotide mixture was used in these experiments). The embryos were frozen just before the onset of gastrulation (stage 9.5) and assayed for induction of the Wnt pathway target gene *Xnr3* by northern hybridization. Injection of Wnt8b RNA into

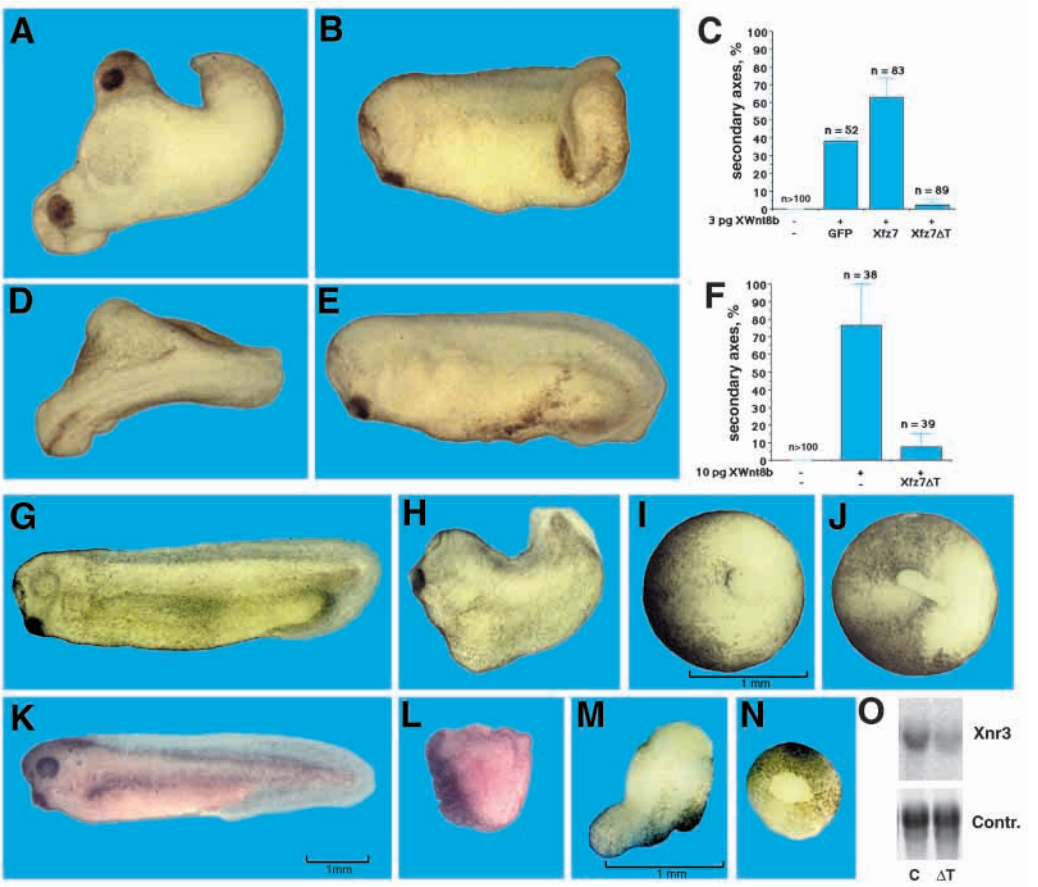

Fig. 6. Generation of dominant-negative Xfz7. Embryos shown are at tailbud stages. (A-C) Wnt8b synergizes with Xfz7 in the secondary axis induction while the truncated form of Xfz7 (Xfz7ΔT) does not. (A) Wnt8b+Xfz7-injected embryo. (B) Wnt8b+Xfz7ΔT-injected embryo. (C) Summary of axis formation assays using moderate Wnt8b dose. (D-F) Xfz7ΔT antagonizes Wnt8b ability to induce secondary axes. (D) Wnt8b alone-injected embryo. (E) Wnt8b+Xfz7ΔT-injected embryo. (G,I,K,M) Uninjected control embryos. (G,H) Overexpression of Xfz7ΔT in an embryo results in shortened embryos with dorsally bent tails. (I,J) Overexpression of Xfz7ΔT in an embryo results in abnormally elongated blastopores. (K,L) Overexpression of Xfz7ΔT in an oocyte results in severely shortened embryos with very little or no axial structures. (M,N) Overexpression of Xfz7ΔT in an embryo blocks elongation of marginal zones dissected at mid-blastula stage. (O) Northern analysis of a dorsal organizer marker Xnr3 expression in Xfz7ΔT expressing embryos. Note that Xnr3 is significantly reduced in ΔT-injected embryos.

control oocytes resulted in strong induction of *Xnr3*, while injection of Wnt8b into Xfz7-depleted oocytes resulted in the reduced level of *Xnr3* expression compared to uninjected controls (Fig. 5E). Indeed, these embryos displayed no ability to respond to ectopic Wnt signaling, as assayed by *Xnr3* induction. To test if Xfz7 functions upstream of β-catenin, we injected β-catenin RNA into Xfz7-depleted oocytes and assayed for the induction of *Xnr3*. In contrast to the Wnt8b results, injection of β-catenin fully restored *Xnr3* expression in Xfz7-depleted oocytes (Fig. 5F). These results place endogenous Xfz7 function between an axis-inducing Wnt and β-catenin in the dorsal mesoderm-induction pathway (Fig. 5G).

Generation of dominant-negative Xfz7

To test the requirement of the putative Xfz7 cytoplasmic tail for fz-mediated wnt signal transduction, we generated an altered form of Xfz7, which lacked the last 25 amino acid residues in the cytoplasmic tail (Xfz7ΔT). We injected a low dose of ectopic Wnt8b with wild-type Xfz7, Xfz7ΔT or control GFP RNA into a ventral blastomere at the 4-cell stage and scored the injected embryos at tailbud stages for ectopic axes. As expected, adding Xfz7 increased the percentage of embryos with a secondary axis (Fig. 6A-C). Adding Xfz7ΔT, however, did not result in the higher percentage of embryos with secondary axes (Fig. 6A-C). The percentage of the embryos with secondary axes was indeed lower than in the case of injecting Wnt8b with inert GFP RNA. To specifically test for the antagonistic effect of Xfz7ΔT, we injected a higher amount of Wnt8b RNA with or without Xfz7ΔT RNA into a ventral blastomere and scored for secondary axis formation. Addition of Xfz7ΔT greatly reduced but did not abolish the ability of these embryos to form a secondary axis (Fig. 6D-F), demonstrating an antagonistic function of this truncated form of Xfz7. The cytoplasmic tail of Xfz7 is therefore required for Xfz7-mediated wnt signal transduction.

We tested the effects of the addition of this antagonistic form of Xfz7 on embryonic development. Injection of Xfz7ΔT-encoding mRNA resulted in embryos with abnormally elongated blastopores during gastrulation (Fig. 6I,J). Overexpression of Xfz7ΔT caused inhibition of morphogenetic movements as seen by the inhibition of marginal zones elongation explanted at the blastula stage (Fig. 6M,N). Xfz7ΔT-overexpressing embryos developed into shortened embryos with dorsally bent tails (Fig. 6G,H), similar to the ones resulting from overexpressing of the wild-type Xfz7 (see Fig. 2C,D). It may seem surprising that overexpression of wild-type Xfz7 and Xfz7ΔT yields similar phenotypes. However, it is known that Dfz1 overexpression can mimick loss of function in *Drosophila* (Krasnow and Adler, 1994). The mechanism of this effect is not known but may include the titration of ligand at high doses by wild-type fz protein. At equivalent low doses of Xfz7 and Xfz7ΔT mRNA, only Xfz7ΔT effectively causes the bent back phenotype.

To test if Xfz7ΔT could block endogenous Xfz7 signal transduction when expressed very early in development, we injected Xfz7ΔT into oocytes, which were later fertilized using the host-transfer method. Such overexpression of Xfz7ΔT resulted in a more severe phenotype where embryos were extremely shortened, did not close neural folds properly and had no identifiable heads or dorsal mesoderm structures (Fig. 6K,L). This phenotype correlated with the reduced expression of dorsal

organizer gene *Xnr3*, as analyzed by northern hybridization (Fig. 5O). These results support the idea that Xfz7 functions very early in development in dorsal mesoderm induction.

DISCUSSION

Evidence for a conserved role for Wnt signaling in vertebrate axis induction

The data provided in this article strongly argue for the requirement of Wnt signaling in *Xenopus* dorsoventral axis formation. The axis-induction mechanism, as well as many other developmental events, is quite likely to be evolutionally conserved. A dominant negative frizzled molecule can block dorsal organizer formation in zebrafish (Nasevicius et al., 1998). This study implicated fz signaling in axis formation but, due to the limitations of the methodology, did not identify the endogenous fz protein encoding such a function. Furthermore, *Wnt3* knockout mice do not form a primitive streak, mesoderm or node (Liu et al., 1999), demonstrating that wnt pathway is required for murine axis specification. Our results argue that primary embryonic axis induction is highly conserved between frogs, fish and mice.

When and how does Xfz7 get activated?

The question of what is the ligand for Xfz7 still remains open. Each maternally encoded wnt gene that we tested was capable, when expressed at sufficiently high levels, of activating *Xfz7* for dorsoventral mesoderm specification. Consequently, we cannot exclude any of these as likely ligands. The only maternal Wnt protein known to be asymmetrically distributed along the dorsoventral axis is Wnt11 (Schroeder et al., 1999), which makes it a likely candidate for the endogenous axis induction. Alternatively, we have found in *Xenopus* a maternally expressed mouse Wnt3 homolog (S. S. and S. C. E., unpublished data). Future experiments will have to determine the actual ligand for Xfz7.

Our results demonstrate that Xfz7 functions before the mid-blastula transition in axis specification, and we do not really know more precisely when its function is required. Since we show that β-catenin functions downstream of Xfz7, Xfz7 is likely to transduce a signal even earlier than β-catenin. β-catenin has been shown to be differentially localized to nuclei in *Xenopus* embryos by the 32-cell stage and in the cytoplasm as early as the 2-cell stage of development (Larabell et al., 1997). If β-catenin is solely regulated by Xfz7 activation, then Xfz7 has to function very early in development.

How can *Xfz7* be specifically activated in *Xenopus* this early in embryonic development? An example of wnt pathway functioning at the 4-cell stage in orienting a mitotic spindle comes from *C. elegans* (Schlesinger et al., 1999). In *Xenopus* there are no distinct embryonic regions formed yet at this stage of development, yet autocrine or paracrine wnt signaling might participate in polarizing the dividing cells by activating receptors on a particular side of a cell as it is seen in *Drosophila* wing hair induction (Krasnow et al., 1995).

Alternatively, *Xfz7* may be activated ubiquitously in an embryo, possibly even by a number of maternally expressed Wnts. If at least a single downstream component of the pathway is localized asymmetrically, as is known to be the case for Dishevelled (Miller et al., 1999), the ubiquitous activation of *Xfz7* would result in a localized signal transduction. This model is actually in agreement with asymmetry established by cortical rotation data. So, according to Miller et al. (1999), cortical rotation would result in the dorsal enrichment of Dsh. An inactivated Dsh may not be able to effectively transduce the signal. Ubiquitous Wnt signaling through Xfz7 would result in ubiquitous Dsh activation. But, since Dsh is already enriched dorsally due to cortical rotation, higher signaling would be transmitted on the dorsal side, resulting in the dorsal enrichment of β-catenin.

We cannot exclude the possibility that β-catenin could also be regulated by another, Xfz7-independent pathway. The strongest phenotypes observed in Xfz7-depleted embryos, however, argue that this putative alternative pathway is not always sufficient for endogenous axis specification. Analysis of a strong loss-of-function phenotype for Xfz7 ligand(s) could potentially distinguish between these single and dual signal models.

The very early requirement for Xfz7 function may explain why previous attempts to block Wnt signaling at the ligand or receptor level have failed. This is supported by our findings that the Fz7 truncation ΔT had to be injected very early (oocytes) to cause ventralization; similar doses injected later into embryos caused other defects, presumably due to interference with zygotic Wnt pathways, but did not cause ventralization.

We thank M. O'Connor, J. Lohr, M. Kofron and anonymous reviewers for critical comments on this manuscript. This work was supported by the Minnesota Medical Foundation and the National Institutes of Health (R01GM55877 to S. C. E. and R01HD33002 to J. H.).

REFERENCES

Bhanot, P., Brink, M., Samos, C. H., Hseih, J.-C., Wang, Y., Macke, J. P., Nathans, J. and Nusse, R. (1996). A new member of the *frizzled* family from *Drosophila* functions as a *Wingless* receptor. *Nature* **382**, 225-230.

Bhat, K. M. (1998). frizzled and frizzled 2 play a partially redundant role in wingless signaling and have similar requirements to wingless in neurogenesis. *Cell* **95**, 1027-1036.

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.

Cho, K. W., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene goosecoid. *Cell* **67**, 1111-1120.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt-8, a *Xenopus* Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1055.

Christian, J. L. and Moon, R. T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of *Xenopus*. *Genes Dev.* **7**, 13-28.

Cui, Y., Brown, J. D., Moon, R. T. and Christian, J. L. (1995). Xwnt-8b: a maternally expressed *Xenopus* Wnt gene with a potential role in establishing the dorsoventral axis. *Development* **121**, 2177-2186.

Darras, S., Marikawa, Y., Elinson, R. P. and Lemaire, P. (1997). Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser. *Development* **124**, 4275-4286.

Deardorff, M. A., Tan, C., Conrad, L. J. and Klein, P. S. (1998). Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* **125**, 2687-2700.

Dominguez, I., Itoh, K. and Sokol, S. Y. (1995). Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc. Natl Acad. Sci. USA* **92**, 8498-8502.

Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L. and Moon, R. T. (1995). Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in *Xenopus* embryos. *Molec. Cell. Biol.* **15**, 2625-2634.

Fan, M. J., Gruning, W., Walz, G. and Sokol, S. Y. (1998). Wnt signaling and

transcriptional control of Siamois in *Xenopus* embryos. *Proc. Natl Acad. Sci. USA* **95**, 5626-5631.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in *Xenopus*. *Nature* **389**, 517-519.

Gradl, D., Kuhl, M. and Wedlich, D. (1999). Keeping a close eye on Wnt-1/wg signaling in *Xenopus. Mech. Dev.* **86,** 3-15.

Gurdon, J. B., Fairman, S., Mohun, T. J. and Brennan, S. (1985). Activation of muscle-specific actin genes in *Xenopus* development by an induction between animal and vegetal cells of a blastula. *Cell* **41**, 913-922.

Harland, R. M. (1997). In situ hybridization. In *Early Development of Xenopus laevis.* (ed. H. L. Sive, R. M. Grainger and R. M. Harland), pp. 93-103. Cold Spring Harbor: Cold Spring Harbor Press.

Harris, J., Honigberg, L., Robinson, N. and Kenyon, C. (1996). Neuronal cell migration in *C. elegans*: regulation of Hox gene expression and cell position. *Development* **122**, 3117-3131.

He, X., Saint-Jeannet, J. P., Wang, Y., Nathans, J., Dawid, I. and Varmus, H. (1997). A member of the Frizzled protein family mediating axis induction by Wnt-5A. *Science* **275**, 1652-1654.

He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. and Dawid, I. B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* **374**, 617-622.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y. and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* **79**, 791-803.

Herman, M. A. and Horvitz, H. R. (1994). The *Caenorhabitis elegans* gene *lin-44* controls the polarity of asymmetric cell divisions. *Development* **120**, 1035-1047.

Hoppler, S., Brown, J. D. and Moon, R. T. (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos. *Genes Dev.* **10**, 2805-2817.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409-3417.

Hyatt, T. and Ekker, S. C. (1999). Vectors and techniques for ectopic gene expression in zebrafish. In *The Zebrafish*, vol. 1 (ed. H. W. Detrich, L. I. Zon and M. Westerfield). San Diego: Academic Press.

Itoh, K., Tang, T. L., Neel, B. G. and Sokol, S. Y. (1995). Specific modulation of ectodermal cell fates in *Xenopus* embryos by glycogen synthase kinase. *Development* **121**, 3979-3988.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus* laevis embryos. *Dev. Biol.* **127**, 64-77.

Kennerdell, J. R. and Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**, 1017-1026.

Kelly, G.M., Eib, D.W., and Moon, R.T. (1991). Histological preparation of *Xenopus laevis* oocytes and embryos. In *Methods In Cell Biology* **36,** 389-417.

Krasnow, R. E., Wong, L. L. and Adler P. N. (1995). dishevelled is a component of the frizzled signaling pathway in *Drosophila*. *Development* **121**, 4095-4102.

Krasnow R. E. and Adler P. N. (1994). A single frizzled protein has a dual function in tissue polarity. *Development* **120**, 1883-1893.

Ku, M. and Melton, D. A. (1993). *Xwnt-11*: a maternally expressed *Xenopus* wnt gene. *Development* **119**, 1161-1173.

Larabell, C. A., Torres, M., Rowning, B. A., Yost, C., Miller, J. R., Wu, M., Kimelman, D. and Moon, R. T. (1997). Establishment of the dorsoventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. *J. Cell Biol.* **136**, 1123-1136.

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.

Leyns, L., Bouwmeester, T., Kim, S.-H., Piccolo, S. and De Robertis, E. M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. *Cell* **88**, 747-756.

Liu, P., Wakamiya, M., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nature Genetics* **22,** 361-365.

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.

Miller, J. R., Rowning, B. A., Larabell, C. A., Yang-Snyder, J. A., Bates, R. L. and Moon, R. T. (1999). Establishment of the dorsal-ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of dihevelled that is dependent on cortical rotation. *J. Cell Biol.* **146,** 427-437.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Moon, R. T., Brown, J. D. and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.

Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S. (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* **119**, 97-111.

Muller, H., Samanta, R. and Wieschaus, E. (1999). Wingless signaling in the *Drosophila* embryo: zygotic requirements and the role of the frizzled genes. *Development* **126**, 577-586.

Nasevicius, A., Hyatt, T., Kim, H., Guttman, J., Walsh, E., Sumanas, S., Wang, Y. and Ekker, S. C. (1998). Evidence for a frizzled-mediated wnt pathway required for zebrafish dorsal mesoderm formation. *Development* **125**, 4283-4292.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* **121**, 755-765.

Pillemer, G., Yelin, R., Epstein, M., Gont, L., Frumkin, Y., Yisraeli, J. K., Steinbeisser, H. and Fainsod, A. (1998). The Xcad-2 gene can provide a ventral signal independent of BMP-4. *Mech. Dev.* **74**, 133-143.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.-H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.

Sawa, H., Lobel, L. and Horvitz, H. R. (1996). The Caenorhabditis elegans gene lin-17, which is required for certain asymmetric cell divisions, encodes a putative seven-transmembrane protein similar to the *Drosophila* frizzled protein. *Genes Dev.* **10**, 2189-2197.

Scharf, S. R. and Gerhart, J. C. (1983). Axis determination in eggs of *Xenopus* laevis: a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation. *Dev. Biol.* **99**, 75-87.

Schlesinger, A., Shelton, C. A., Maloof, J. N., Meneghini, M. and Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early Caenorhabditis elegans embryo without requiring gene transcription in the responding cell. *Genes Dev.* **13**, 2028-38.

Schroeder, K. E., Condic, M. L., Eisenberg, L. M. and Yost, H. J. (1999). Spatially regulated translation in embryos: asymmetric expression of maternal Wnt-11 along the dorsal-ventral axis in *Xenopus. Dev. Biol.* **214,** 288-297.

Shi, D.-L., Goisset, C. and Boucaut, J.-C. (1998). Expression of Xfz3, a *Xenopus* frizzled family member, is restricted to the early nervous system. *Mechan. Dev.* **70**, 35-47.

Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413.

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. (1996). Analysis of Dishevelled signaling pathways during *Xenopus* development. *Current Biol.* **6**, 1456-1467.

Thorpe, C. J., Schlesinger, A., Clayton Carter, J. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* bBlastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.

Wang, S., Krinks, M., Lin, K., Luyten, F. P. and Moos Jr., M. (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* **88**, 757-766.

Wang, Y., J.P., M., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1996). A large family of putative transmembrane receptors homologous to the product of the Drosphila tissue polarity gene frizzled. *J. Biol. Chem.* **7**, 1-9.

Wesley, C. S. (1999). Notch and Wingless regulate expression of cuticle patterning genes. *Mol. Cell Biol.* **19**, 5743-5758.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C.-L. and Moon, R. T. (1996). A *frizzled* homolog functions in a vertebrate *Wnt* signaling pathway. *Current Biol.* **6**, 1302-1306.

Yost, C., Farr, G. H., 3rd, Pierce, S. B., Ferkey, D. M., Chen, M. M. and Kimelman, D. (1998). GBP, an inhibitor of GSK-3, is implicated in *Xenopus* development and oncogenesis. *Cell* **93**, 1031-1041.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., Lee, J. J., Tilghman, S. M., Gumbiner, B. M. and Constantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181-192.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. *Cell* **94**, 515-524.