Frizzled receptor dimerization is sufficient to activate the Wnt/β-catenin pathway

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Summary

Wnt signaling has an important role in cell-fate determination, tissue patterning and tumorigenesis. Wnt proteins signal through seven-pass transmembrane receptors of the frizzled family to activate β-catenin-dependent transcription of target genes. Using early *Xenopus* embryos, we show that frizzled receptors can dimerize and that dimerization is correlated with activation of the Wnt/β-catenin pathway. Co-immunoprecipitation studies revealed that the receptor Xfz3 exists as a dimer when expressed in *Xenopus* embryos, and it has been shown to activate the Wnt/β-catenin pathway as revealed by expression of the target gene *siamois*. Xfz3 dimerization requires intramolecular and/or intermolecular disulfide linkages, and the N-terminal extracellular region of the receptor, including the cysteine-rich domain (CRD), is sufficient for dimerization. The receptor Xfz7 behaves differently from Xfz3 when overexpressed in the embryo as Xfz7 is monomeric and is unable to directly activate the Wnt/β-catenin pathway. However, activation of this pathway can be achieved by artificially forcing Xfz7 dimerization. These results provide the first direct evidence for the dimerization of frizzled receptors and suggest that dimerization contributes to transducing the Wnt/β-catenin signal.

Key words: Frizzled, Wnt, Dimerization, *Xenopus*

Introduction

The Wnt family of secreted glycoproteins regulates a large number of developmental processes including cell growth, cell polarity and tissue specification. Wnts are crucial cell signaling molecules during development, as well as in adult life, and deregulation of their signaling pathway has major oncogenic effects (Cadigan and Nusse, 1997; Miller et al., 1999). The current data suggest that Wnts signal through seven-transmembrane receptors of the frizzled family (Bhanot et al., 1996). Frizzleds are required for Wnt signaling in vivo, as depletion of both *Drosophila* frizzled 1 and 2 (Dfz1 and Dfz2) results in wingless-like phenotypes (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Kennerdell and Carthew, 1998). Furthermore, Wnts can bind to soluble forms of the frizzled proteins (Hsieh et al., 1999a; Hsieh et al., 1999b; Wang et al., 1997), which may act as a dominant-negative form of the receptor (Cadigan et al., 1998; Hsieh et al., 1999a; Hsieh et al., 1999b; Wang et al., 1997; Zhang and Carthew, 1998). Frizzled proteins share common structural motifs: a putative N-terminal signal sequence, a conserved cysteine-rich domain (CRD), a hydrophilic and highly divergent linker region, seven transmembrane segments and a variable intracellular C-terminal domain. The CRD is believed to act as the Wnt binding site for the receptor. Indeed, in *Drosophila*, the CRD of Dfz2 is necessary and sufficient for binding the wingless ligand (Cadigan et al., 1998; Rulifson et al., 2000; Zhang and Carthew, 1998). Several groups have shown that co-receptors for frizzleds are essential for Wnt signaling (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). They are related to the low-density-lipoprotein (LDL) receptor family and named LRP5 and LRP6. LRP6 is able to bind Wnt-1 and to associate with the Fz8 CRD in a Wnt-dependent fashion (Tamai et al., 2000).

A crucial function of the Wnt pathway is to activate β-catenin-dependent transcription. In the absence of Wnt receptor activation, the modular protein Axin provides a scaffold for the binding of glycogen synthase kinase-3β (GSK3), adenomatous polyposis coli protein (APC) and β-catenin. This, in turn, facilitates β-catenin phosphorylation by GSK3 (Ikeda et al., 1998; Itoh et al., 1998) and leads to the rapid degradation of β-catenin via the ubiquitin pathway (Aberle et al., 1997). Upon Wnt's binding of the frizzled receptor, the Axin–GSK3–APC–β-catenin complex is disrupted by a process involving the cytoplasmic protein Dishevelled (Dsh), and dephosphorylation and recruitment of Axin to LRP5, which is associated with Axin destabilization (Mao et al., 2001; Smalley et al., 1999; Yamamoto et al., 1999). Subsequently, the β-catenin is no longer targeted for ubiquitin degradation and has been shown to accumulate in the nuclei (Fagotto et al., 1998; Yost et al., 1996), where it may interact with members of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) classes of transcription factors to regulate the expression of target genes such as *siamois* (Brannon et al., 1997; Carnac et al., 1996; Lemaire et al., 1995; Molenaar et al., 1996). In *Xenopus*, Wnt/β-catenin signaling plays a crucial role in dorso-ventral axis specification. Ventral overexpression of certain Wnts or frizzleds, Dsh or β-catenin ectopically activates *siamois* transcription and leads to the generation of a complete secondary axis (Moon and Kimelman, 1998; Sokol, 1999). In normal embryos, β-catenin protein accumulates in

the cytoplasm and nuclei of dorsal blastomeres during early cleavage stages (Larabell et al., 1997). Moreover, overepression of GSK3 and Axin or depletion of maternal β-catenin RNA causes deficiencies in dorsal structures (He et al., 1995; Heasman et al., 1994; Yost et al., 1998; Zeng et al., 1997).

The biochemical mechanisms by which the binding of the Wnt ligand to its frizzled receptor elicits signal transduction within the cell are poorly characterized. Numerous studies have suggested that several G-protein-coupled receptor (GPCR) families exist as dimers or even higher structures (Devi, 2001; Gouldson et al., 2000; Hebert and Bouvier, 1998; Milligan, 2001). Recently, biophysical methods based on luminescence and fluorescence energy transfer have confirmed the existence of such oligomeric complexes in living cells (Angers et al., 2000; Angers et al., 2001; Kroeger et al., 2001; Overton and Blumer, 2000). However, whether dimerization is a general property of this class of receptors and whether this is functionally relevant for signal transduction remains controversial (Cvejic and Devi, 1997; George et al., 1998; Gouldson et al., 1998; Hebert et al., 1998; Marshall et al., 1999). In several cases, receptors appear to fold as constitutive dimers shortly after biosynthesis, whereas ligand-promoted dimerization at the cell surface has been proposed for others (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Dimerization is required for normal functioning of β-adrenergic receptors and has been shown to rescue the function of mutant forms of β-adrenergic and angiotensin type I receptors (Hebert et al., 1998).

In this report, we address the question of the potential dimerization property of two *Xenopus* frizzled receptors and the potential role of this dimerization in Wnt/β-catenin signal transduction. Taking advantage of the differential ability of Xfz3 and Xfz7 to activate the Wnt/β-catenin pathway when overexpressed in the *Xenopus* embryo (Umbhauer et al., 2000a), we carried out a comparative study of Xfz3 and Xfz7, which revealed an unexpected correlation between the presence of frizzled dimers and activation of the Wnt/β-catenin pathway. We show that Xfz3 but not Xfz7 exists as a homodimer when expressed in the *Xenopus* embryo and activates transcription of the Wnt/β-catenin target gene *siamois*. Moreover, forced homodimerization of Xfz7 is sufficient for activation of the Wnt/β-catenin pathway, even in the absence of the CRD ligand-binding domain. We therefore propose that dimerization is a crucial step in the transduction of the Wnt/β-catenin signal by frizzled receptors.

Materials and Methods

Plasmid constructs

All constructs (Fig. 1) are cloned in pCS2+ vector (Turner and Weintraub, 1994). Myc-tagged version of wild-type Xfz3 (Xfz3-myc) and Xfz3ΔC (Xfz3ΔC-myc) has been previously described (Umbhauer et al., 2000a). Myc-tagged Xfz7 (Xfz7-myc) was made by insertion of an *Eco*RI/*Not*I digested PCR fragment of Xfz7 cDNA into pCS2-Xfz3-myc digested by *Eco*RI and *Not*I. The PCR fragment was amplified from pCS2-Xfz7 (Djiane et al., 2000) using the upstream primer 5′-TAGGAATTCCCAGCAGTACCACGGG-3′ and the T3 primer.

To obtain Xfz3-HA, the coding sequence of Xfz3 was PCR amplified with an *Eco*RV site added at the C-terminus and cloned in frame with two HA epitopes. To obtain Xfz7ΔC-flag, a *Bam*HI/*Cla*I digested fragment corresponding to the 526 first amino acids of Xfz7 was inserted in frame in the pCS2-extraXfz7-flag (Djiane et al., 2000) digested by *Bam*HI and *Cla*I. To make a myc version of the N-terminal extracellular region of Xfz3 (extra3-myc), a nontagged construct corresponding to the first 196 amino acids of Xfz3 was first cloned in pSP64T (Krieg and Melton, 1987). Then, a *Xho*I/ *Xba*I fragment was inserted in pCS2-Xfz3-myc digested with *Xho*I and *Xba*I. To obtain extra3-flag, pCS2-extraXfz7-flag (Djiane et al., 2000) was digested by *Bam*HI and *Cla*I to replace extraXfz7 with a PCR fragment corresponding to the first 196 amino acids of Xfz3. The antisense primer was 5′-TGAATCGATAGGCAAAGGAGAG-3′ and the sense was the SP6 primer.

The Xfz7-BD-myc construct was obtained by *Eco*RI/*Bgl*II digestion of pCS2-Xfz7-myc and insertion of an *Eco*RI/*Bgl*II-digested PCR fragment amplified from TELmod vector (Lopez et al., 1999). The upstream primer was: 5′-CGGGAATTCGCGCTTGCAGCCAA-TT-3′; the downstream primer was: 5′-GCAAGATCTGCTGAAGG-AGTTCATAGAG-3′.

The fibroblast growth factor receptor-1 (FGFR-1) fusion proteins were obtained as follows: for extra3-R1, the coding sequence for the transmembrane and intracellular domains of FGFR-1 (Friesel and Dawid, 1991) was PCR amplified (upstream primer, 5′-CGGAGCT-CCAACTGGAAATT-3′; downstream primer, 5′-ATTATCTAGATC-AGCGTTTTTTAAG-3′), digested by *Sac*I and *Xba*I and inserted into pCS2-Xfz3-myc digested by the same enzymes. For Xfz3ΔC-R1, the intracellular region of FGFR-1 was PCR amplified (upstream primer, 5′-CGCTTCGAACACCCGTCGAAG-3′; downstream primer, 5′-ATTATCTAGATCAGCGTTTTTTAAG-3′), digested by *Bst*BI and *Xba*I and inserted into pCS2-Xfz3-myc digested by the same enzymes. Torso-FGFR-1 has been previously described in (Umbhauer et al., 2000b). The wild-type FGFR-1 is as in Amaya et al. (Amaya et al., 1991).

All constructs were checked by sequencing. Plasma membrane subcellular localization in blastula animal cap cells was verified by immunodetection and confocal microscopy for all myc and HA tagged constructs bearing a transmembrane domain.

Xenopus embryos and mRNA microinjections

Xenopus eggs were obtained from females injected with 500 IU of human chorionic gonadotropin (Sigma), and artificially fertilized. Eggs were dejellied with 2% cysteine hydrochloride (pH 7.8) and embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Capped mRNAs were synthesized from linearized plasmids using SP6 RNA polymerase (Boehringer Mannheim) in the presence of 500 μM 5′-mGpppG-3′ cap analog, 500 μM each rUTP, rATP, rCTP and 50 μM rGTP. Synthetic mRNA was purified using a Sephadex G-50 column (Pharmacia, Les Ulis, France) and recovered by ethanol precipitation. Microinjection of embryos was performed in 0.1× MBS (Modified Barth's Solution) containing 3% Ficoll 400 using a PLI-100 reproducible pico-injector (Medical Systems, Greenvale, NY). Unless specified, a volume of 10 nl corresponding to 250 pg of synthetic mRNA was injected into each blastomere at the animal pole region of two-cell-stage embryos.

RT-PCR

Animal cap explants from control and injected embryos were dissected at mid-blastula stage (stage 8) and cultured to early gastrula stage (stage 10.5). Extraction of total RNA was as previously described (Umbhauer et al., 2000a). About 5 μg of total RNA were reverse-transcribed in the presence of 100 IU of MLV (Murine Leukemia Virus) reverse transcriptase (Life Technologies, Invitrogen, Cergy-Pontoise, France). In each experiment, 5 μg of total RNA from whole embryos treated the same way but without reverse transcriptase were used as a control for PCR specificity. One tenth of the reverse-

transcribed cDNA was used for PCR amplification in a 25 μl reaction mixture consisting of 1× PCR buffer (Perkin Elmer Cetus, Life Science, Courtaboeuf, France), dNTP at 0.2 mM each, 25 pmol of sense and antisense primers and 0.3 IU of Taq DNA polymerase (Perkin Elmer Cetus). PCR primers were the following: *Siamois* (Lemaire et al., 1995) (5′-AAGGAACCCCACCAGGATAA-3′ and 5′-TACTGGTGGCTGGAGAAATA-3′; 30 cycles); *Xbra* as in Henry et al. (Henry et al., 1996), 30 cycles; and *ornithine decarboxylase* (*ODC*) (Bassez et al., 1990) (5′-GTCAATGATGGAGTGTATGGATC-3′ and 5′-TCCATTCCGCTCTCCTGAGCAC-3′; 26 cycles). PCR products were resolved on a 2% agarose gel containing 1 μg/ml ethidium bromide.

Western blotting and immunoprecipitation

Embryos at the early gastrula stage were extracted in lysis buffer [10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% aprotinin (Sigma), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% leupeptin (Sigma)]. After 10 minutes centrifugation at 13,200 ***g*** at 4°C, supernatants were diluted in 2× Laemmli buffer without β-mercaptoethanol for direct western blotting analysis. For these analyses, samples of protein extracts from the equivalent of 0.5 embryos were fractionated by electrophoresis on SDS-polyacrylamide gels according to Laemmli (Laemmli, 1970). For immunoprecipitation experiments, extracted proteins from an equivalent of ten embryos were separated in two equal parts and processed for immunoprecipitation using an anti-c-myc (9E10, Santa Cruz, Santa Cruz, CA), an anti-influenza-HA (12CA5, Boehringer Mannheim) or an anti-flag (M2, Sigma) monoclonal antibody. Immunoblotting analyses were performed after gel electrophoresis in reducing conditions and electrotransfer of separated proteins on nitrocellulose sheets (HYBON-C, Amersham, Les Ulis, France). The membranes were incubated in the presence of a 1/1000 dilution of monoclonal antibodies 9E10 or 12CA5 or in the presence of a 1/500 dilution of the M2 antibody. After incubation with horseradish peroxidase-conjugated secondary antibody (Immunotech Beckman Coulter Company, Maseille, London, 1/5000 dilution), bands were visualized using the ECL chemiluminescence kit (Amersham).

Results

Xfz3 forms homodimers in *Xenopus* embryos

To analyze the biochemical properties of the Xfz3 receptor, we have constructed tagged-versions of the wild-type Xfz3 (Xfz3-HA, Xfz3-myc) and of a C-terminal truncated form, which retains five amino acids after the seventh transmembrane domain (Xfz3ΔC-myc) (Fig. 1) (Shi et al., 1998; Umbhauer et al., 2000a). These molecules were overexpressed in *Xenopus* embryos and analyzed either by western blot or immunoprecipitation. Embryos at the two-cell stage were injected with mRNA encoding the tagged receptors and cultured until the early gastrula stage when protein extracts were prepared. Under nonreducing conditions, direct western blot analysis of Xfz3-myc allows for the consistent detection of not only a band at approximately 90-95 kDa, which corresponds to the anticipated weight of the monomeric receptor, but also a band of a potentially dimeric species at 190 kDa (Fig. 2A). Immunoblotting of the truncated form Xfz3ΔC-myc reveals bands of approximately 65-75 kDa as expected for the monomer and an additional band at 130-150 kDa. These data are consistent with the idea that the form with higher molecular weight represents a homodimer. In some cases, discrete bands, which could represent trimeric or even higher order structures, were observed (Fig. 2A, star). Similar results

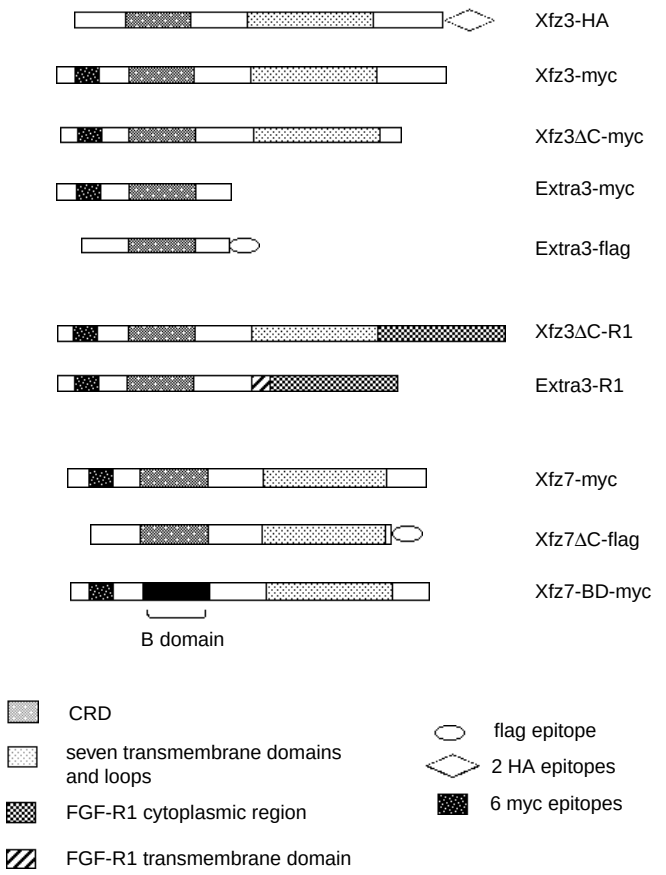

Fig. 1. Structure of Xfz3 and Xfz7 derivatives used in this study. The myc-tagged proteins contain six myc epitopes inserted at the N-terminus immediately after the peptide signal. The flag and HA tags are located at the C-terminus end of the proteins. Xfz3-ΔC-myc corresponds to Xfz3 but retains only five amino acids after the seventh transmembrane domain (Umbhauer et al., 2000a). Extra3-myc and extra3-flag correspond to the 196 first amino acids of Xfz3 bearing myc and flag tags, respectively. Extra3-R1 is a chimeric receptor in which the 196 N-terminal amino acids of Xfz3 are fused with the transmembrane and cytoplasmic domains of FGFR-1 (Musci et al., 1990). Xfz3ΔC-R1 corresponds to the first 505 N-terminal amino acids of Xfz3 fused with the cytoplasmic domain of FGFR-1. Xf7ΔC-flag corresponds to Xfz7 truncated immediately after the seventh transmembrane domain at residue 525. In Xfz7-BD, the CRD of Xfz7 is swapped with the oligomerization domain (B domain) of the ETS protein, TEL (Lopez et al., 1999).

were obtained using an HA rather than a myc epitope tag (Fig. 2B). We performed a competition experiment in which 200 pg of Xfz3ΔC-myc mRNA was co-injected with 800 pg of Xfz3-HA mRNA. Protein extracts were analyzed with the anti-myc antibody by western blotting. Expression of Xfz3-HA led to the disappearance of the 130-150 kDa immunoreactive species representing the dimeric form of Xfz3ΔC-myc. A heavier form, also revealed with the HA antibody (not shown) and which probably corresponds to a Xfz3ΔC-myc/Xfz3-HA heterodimer, appeared (Fig. 2C).

To confirm that the species of higher molecular weight observed in direct western blot corresponded to an Xfz3 homodimer, we devised a differential co-immunoprecipitation strategy. Xfz3-HA, Xfz3ΔC-myc and Xfz3-myc were

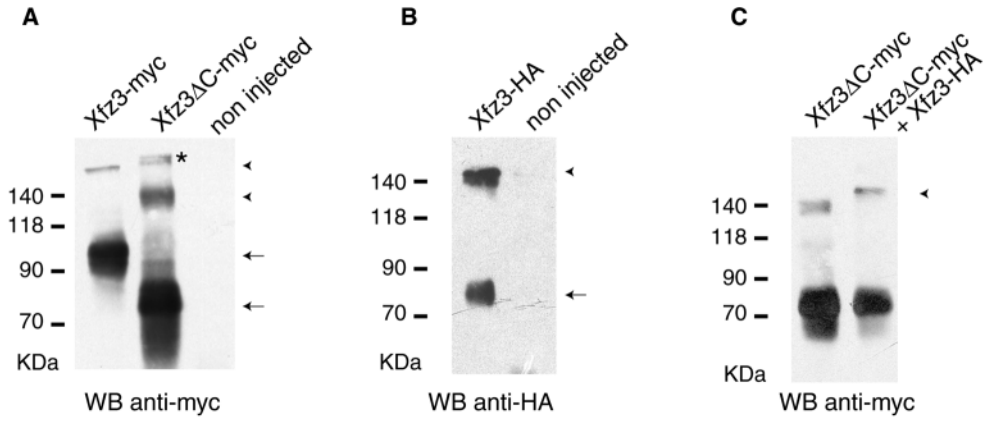

Fig. 2. Xfz3 receptor forms homodimers in *Xenopus* embryo. (A) Embryos were injected at the two-cell stage with mRNA encoding the Xfz3-myc receptor or the C-terminal truncated form Xfz3ΔC-myc. Protein extracts derived from gastrula were immunoblotted following SDS-PAGE under unreduced conditions, using the 9E10 anti-myc antibody. For both constructs, the blot reveals immunoreactive bands corresponding to the expected monomeric form (arrows) as well as a species of higher molecular mass corresponding to twice that of the monomer (arrowhead) or even higher (star). (B) Embryos were injected at the two-cell stage with mRNA encoding Xfz3-HA. Embryonic extracts were immunoblotted using 12CA5 monoclonal antibody as describe in (A). A band corresponding to the expected monomeric form (arrow) plus a higher form corresponding to the expected size for a dimer (arrowhead) are shown. (C) Xfz3ΔC-myc mRNA (200 pg) was injected alone or co-injected with Xfz3-HA mRNA (800 pg). Protein extracts were analyzed with the anti-myc antibody. Co-expression of a high amount of Xfz3-HA leads to the disappearance of the band corresponding to the dimeric form of Xfz3ΔC-myc. A higher band appears (arrowhead), revealing the presence of Xfz3ΔC-myc/Xfz3-HA heterodimer.

expressed either separately or in combination in *Xenopus* embryos. The receptors were then immunoprecipitated with an anti-HA or an anti-myc antibody, subjected to SDS-PAGE and blotted with one or the other antibody. The anti-myc antibody specifically precipitated Xfz3ΔC-myc, whereas the anti-HA antibody did not (Fig. 3, lanes 1 and 2). Immunoprecipitation of protein extracts from embryos co-expressing Xfz3ΔC-myc and Xfz3-HA with the anti-HA antibody resulted in the co-precipitation of Xfz3ΔC-myc (Fig. 3, lane 3). Similar results were obtained for the wild-type Xfz3-HA and Xfz3-myc (Fig. 3, lanes 5 and 6). In both cases, two bands corresponding in size to the monomeric and the dimeric forms of the receptors were co-immunoprecipitated, suggesting that Xfz3 was also present as an oligomer. Overexpression of the same tagged proteins in COS cells yielded identical results (not shown). Taken together, these results show that Xfz3 protein is able to form homodimers when expressed in *Xenopus* embryos.

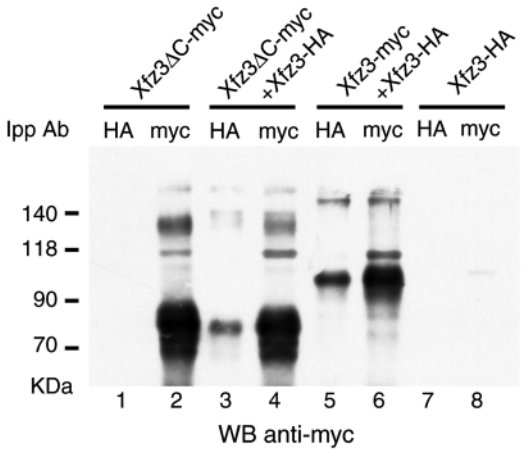

Fig. 3. Co-immunoprecipitation of different epitope-tagged wild-type and truncated Xfz3 receptors. Embryos were injected with the mRNA encoding the indicated receptor (Xfz3-myc or Xfz3ΔC-myc) alone or in combination with Xfz3-HA. Protein extracts from injected embryos were subjected to immunoprecipitation by using the anti-HA antibody (lanes 1, 3, 5, 7) or the anti-myc antibody (lanes 2, 4, 6, 8). Immunoprecipitates were then immunoblotted with the anti-myc antibody. Anti-HA antibody does not immunoprecipitate any myc-positive bands from embryos expressing only Xfz3-HA (lane 7) or Xfz3ΔC-myc (lane 1) but does precipitate bands corresponding to Xfz3ΔC-myc in co-injected embryos (lane 3). Similar results are obtained when Xfz3ΔC-myc is replaced by the full-length Xfz3-myc (lane 5).

Xfz3 dimerization is disrupted by reducing agents such as β-mercaptoethanol

To determine whether disulfide bonds are important for Xfz3 dimer formation, treatment with 2% or 20% β-mercaptoethanol was performed before SDS-PAGE on protein extracts from embryos expressing Xfz3ΔC-myc. In parallel, to prevent artificial disulfide bonds, protein extracts were also treated with 10 mM iodoacetamide (IAA). As shown in Fig. 4, β-mercaptoethanol treatment led to a significant diminution of the dimer/monomer ratio. In the IAA-treated extracts, the dimeric form remained, excluding the possibility that the band with high molecular mass was an artefact due to disulfide bond exchange reactions during the preparation of samples. Thus, Xfz3 is able to form a SDS resistant dimer in *Xenopus* embryo and its dimerization relies, at least partly, on disulfide linkages.

The N-terminal extracellular region of Xfz3 is sufficient for dimerization

The N-terminal extracellular region of frizzled receptors contains a conserved CRD interacting with Wnt ligand. We have tested whether this region alone is capable of dimerization when expressed in *Xenopus* embryos. Embryos were injected with mRNA encoding the first 196 N-terminal amino acids of Xfz3 (corresponding to the N-terminal extracellular region including the CRD) bearing either a flag (extra3-flag) or a myc

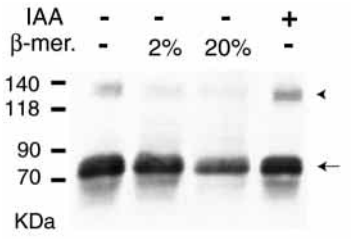

Fig. 4. Xfz3 dimerization is sensitive to β-mercaptoethanol. Protein extracts from embryos overexpressing Xfz3ΔC-myc were analyzed by western blot with the anti-myc antibody. Before SDS-PAGE, protein samples were incubated with 2% or 20% β-mercaptoethanol. This treatment leads to a diminution of the dimer/monomer ratio. The dimeric form of the receptor is still strongly detected in extracts treated with 10 mM iodoacetamide (IAA), which prevents the artificial formation of disulfide bonds during sample preparation.

(extra3-myc) tag. Co-immunoprecipitation experiments were performed using an anti-myc or an anti-flag antibody. In protein extracts derived from embryos expressing both constructs, anti-flag co-immunoprecipitated the extra3-myc protein (Fig. 5A, lane 5), revealing the presence of extra3-myc/extra3-flag dimers. In the embryo, the N-terminal extracellular region of Xfz3 has therefore an intrinsic capacity to form a homodimer. We asked next whether this region was sufficient to induce dimerization when added to a membrane-bound heterologous context. Two chimeric constructs between Xfz3 and the tyrosine kinase FGFR-1 receptor were devised. For one construct (extra3-R1), the extracellular domain of FGFR-1, which contains the FGF ligand binding site, has been replaced by the N-terminal extracellular region of Xfz3 (including the CRD) (Fig. 1). The other construct, Xfz3ΔC-R1, was made by replacing the C-terminal cytoplasmic region of Xfz3 by the intracellular portion of the FGFR-1 receptor. The experimental concept is based on the fact that tyrosine kinase receptors are known to transduce intracellular signaling via dimerization on ligand binding. If dimerization of the chimeric receptors occurs, the Xfz3 part of the chimeric protein should be responsible for this dimerization, as no FGF ligand-binding site is present in these receptors.

The chimeric receptors extra3-R1 and Xfz3ΔC-R1 were overexpressed in *Xenopus* blastula animal cap cells and transcriptional activation of *Xbra* was tested by RT-PCR. *Xbra* is a target gene of the transduction pathway activated by the FGFR-1 in response to FGF ligand in ectoderm animal cap cells. Activation of its transcription will indicate the presence of receptor dimers. As a positive control, we overexpressed a constitutive form of FGFR-1 receptor, torso-R1 (Umbhauer et al., 2000b) and the wild-type FGFR-1 as a negative control. The same quantity of mRNA (50 pg) was injected for each construct. As shown in Fig. 5B, extra3-R1 and Xfz3ΔC-R1 overexpression leads to the transcriptional activation of *Xbra*, as does the torso-R1 positive control. This ability is correlated with phosphorylation on tyrosine residues of these hybrid proteins (Fig. 5C). In the same experiment, *Xbra* was not expressed in response to overexpressing the wild-type FGFR-1 (Fig. 5C). These experiments show that the N-terminal extracellular portion of Xfz3 is sufficient to induce dimerization either in a frizzled receptor or in an unrelated context.

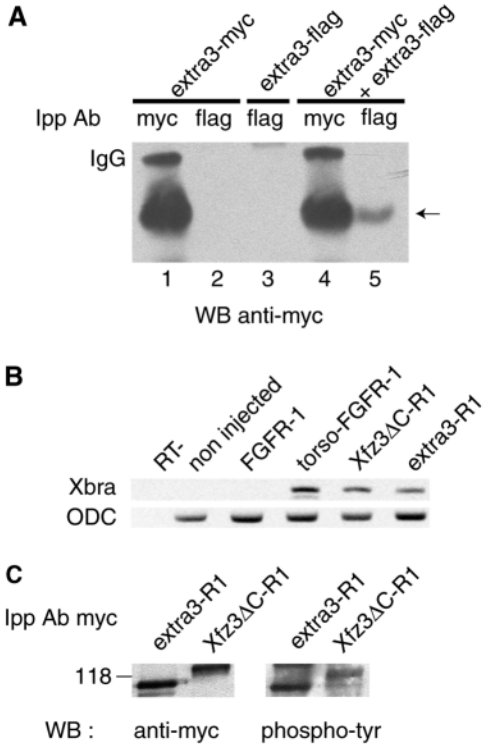

Fig. 5. The N-terminal extracellular region of Xfz3 is sufficient to induce dimerization. (A) Co-immunoprecipition of tagged N-terminal extracellular region of Xfz3. mRNA encoding extra3-myc and extra3-flag were injected alone or in combination in two-cell-stage embryos. Protein extracts from injected embryos were subjected to immunoprecipitation by using the anti-myc antibody (lanes 1 and 4) or the anti-flag antibody (lanes 2, 3 and 5). Immunoprecipitates were then immunoblotted with the anti-myc antibody. Extra3-myc is indicated with an arrow. (B) Analysis of *Xbra* expression in animal caps in response to the wild-type FGFR-1, a constitutive form of FGF-R1 receptor (torso-R1), Xfz3ΔC-R1 or extra3-R1. Total RNA extracted from injected animal caps was assayed for *Xbra* expression at the early gastrula stage by RT-PCR. ODC is a loading control. RT–, control without reverse transcriptase. In each case, 50 pg of synthetic mRNA were injected. (C) Analysis of extra-R1 and Xfz3ΔC-R1 phosphorylation. Embryos were injected with mRNA encoding the myc-tagged extra3-R1 or Xfz3ΔC-R1 proteins. Protein extracts from the early gastrula stage were immunoprecipitated by the anti-myc antibody, separated by SDS-PAGE and blotted on nitrocellulose. Blots were analyzed with either anti-myc or anti-phosphotyrosine (4G10, UBI) antibodies.

Functional correlation between frizzled dimerization and activation of the Wnt/β-catenin pathway

In previous work, we have shown that overexpression of Xfz3 in *Xenopus* blastula animal cap cells is sufficient to activate transcription of the Wnt/β-catenin target gene *siamois*. Overexpression of the receptor Xfz7, however, does not lead to *siamois* expression (Umbhauer et al., 2000a). Using the differential ability of these two receptors to activate the Wnt/β-catenin pathway after overexpression in *Xenopus* embryo, we have addressed the functional significance of frizzled dimerization.

We first analyzed the ability of the receptor Xfz7 to form dimers when overexpressed in *Xenopus* embryos. Western blot

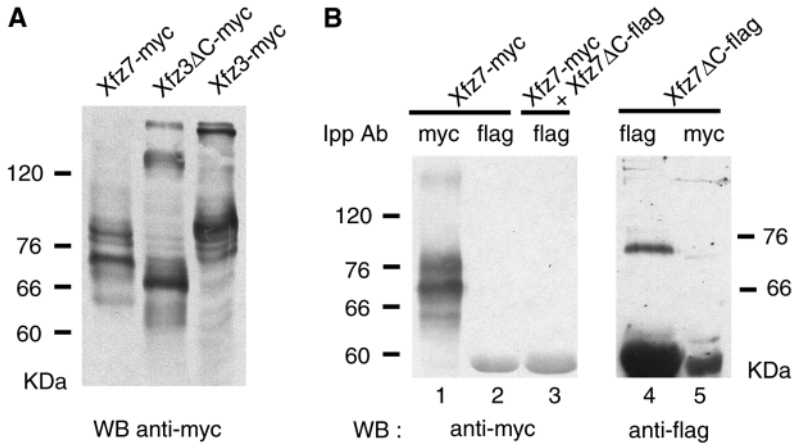

Fig. 6. Dimeric forms of Xfz7 are not detected after overexpression in *Xenopus* embryo. (A) Direct immunoblot analysis using anti-myc antibody of protein extracts from gastrula embryos overexpressing Xfz7-myc, Xfz3ΔC-myc or Xfz3-myc. In protein extracts derived from Xfz7-myc-injected embryos, the anti-myc antibody revealed one group of three major bands corresponding to the expected molecular weight for Xfz7-myc; no band at the size of the putative dimeric form is clearly detected. By contrast, monomeric and dimeric forms of Xfz3-myc and Xfz3ΔC-myc are observed. (B) Embryos were injected with mRNA encoding Xfz7-myc or Xfz7ΔC-flag, or both. Protein extracts from injected embryos were subjected to immunoprecipitation by using the anti-myc antibody (lanes 1 and 5) or the anti-flag antibody (lanes 2, 3 and 4). Immunoprecipitates were then immunoblotted with the anti-myc antibody (left panel) or anti-flag antibody (right panel). Anti-flag antibody does not immunoprecipitate any myc-positive bands from embryos expressing both Xfz7-myc and Xfz7ΔC-flag.

analysis of protein extracts derived from Xfz7-myc-injected embryos revealed only one group of three bands corresponding to the expected molecular weight of the monomeric Xfz7-myc (Fig. 6A). In most cases, no other detectable signal was observed, although in some cases (two of ten), a faint band corresponding to the size of a putative Xfz7-myc dimer was visible. Co-immunoprecipitation experiments confirmed these western blot analyses. The anti-flag antibody did immunoprecipitate Xfz7ΔC-flag (Fig. 6B, lane 4) but it did not co-precipitate Xfz7-myc in protein extracts derived from embryos injected with Xfz7-myc and Xfz7ΔC-flag, (Fig. 6B, lane 3). These results show that, unlike Xfz3, Xfz7 protein is almost solely present in a monomeric form when expressed in the embryo.

Activation of the Wnt/β-catenin pathway in blastula animal cap cells in response to Xfz3 but not Xfz7 may be due to the endogenous expression of a specific ligand for Xfz3. If this is true, co-expression of the N-terminal extracellular region of Xfz3 should block activation of the Wnt/β-catenin pathway in response to Xfz3 by sequestering the ligand in the extracellular compartment. Embryos were injected at the two-cell stage with Xfz7-myc RNA, Xfz3-HA RNA or a combination of Xfz3-HA and extra3-myc RNA. Animal caps were dissected at the blastula stage, cultured to early gastrula and analysed by RT-PCR for *siamois* expression (Fig. 7A). As expected, Xfz3-HA but not Xfz7-myc activated *siamois* expression. Notably, the level of *siamois* expression in caps co-expressing at the same time Xfz3-HA and extra3-myc was very similar to the level obtained in response to Xfz3-HA alone (Fig. 7A). Moreover, co-injection of extra3-myc did not inhibit the formation of Xfz3 dimers, as shown by immunoprecipitation and western blot analysis (Fig. 7B). These results suggest that Xfz3 dimerizes and activates the Wnt/β-catenin pathway in a ligand-independent manner, at least in ectoderm animal cap cells.

These results described above show a correlation between the presence of dimers and the activation of the Wnt/β-catenin pathway. To determine whether activation of the Wnt/β-catenin pathway was indeed directly related to frizzled receptor dimerization, we designed a construct to artificially force the dimerization of the Xfz7 receptor. We interchanged the CRD of Xfz7 with an unrelated oligomerization domain (Xfz7-BD), the B domain of ETS transcription factor TEL (translocated

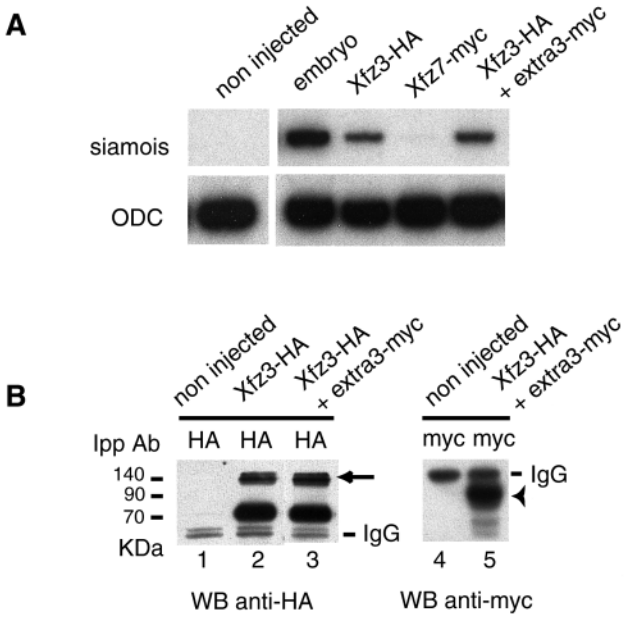

Fig. 7. The N-terminal extracellular domain of Xfz3 is unable to inhibit Xfz3 dimerization and activation of the Wnt/β-catenin pathway in response to overexpression of Xfz3 in *Xenopus* embryo. Embryos were injected with mRNA encoding Xfz7-myc, Xfz3-HA or a combination of Xfz3-HA and extra3-myc mRNA. (A) RT-PCR analysis of the Wnt/β-catenin target gene *siamois* in animal caps cultured to the early gastrula stage. *Siamois* expression in whole embryo is used as a positive control and in noninjected caps as a negative control. *ODC* is a loading control. (B) Immunoprecipitation and western blot analysis of protein extracts from injected embryos. Expression of Xfz3-HA is revealed by immunoprecipitation using the anti-HA antibody followed by western blot analysis with the same antibody. The dimeric form of Xfz3-HA (arrow) is detected after injection of Xfz3-HA mRNA alone (lane 2) or in combination with extra3-myc mRNA (lane3). Expression of the extra3-myc protein in co-injected embryos is checked by immunoprecipitation and western blot with the anti-myc antibody (lane 5, arrowhead).

Ets leukemia) (Lopez et al., 1999). Western blot and immunodetection analyses revealed that this protein indeed migrates as monomeric and dimeric forms, and even higher

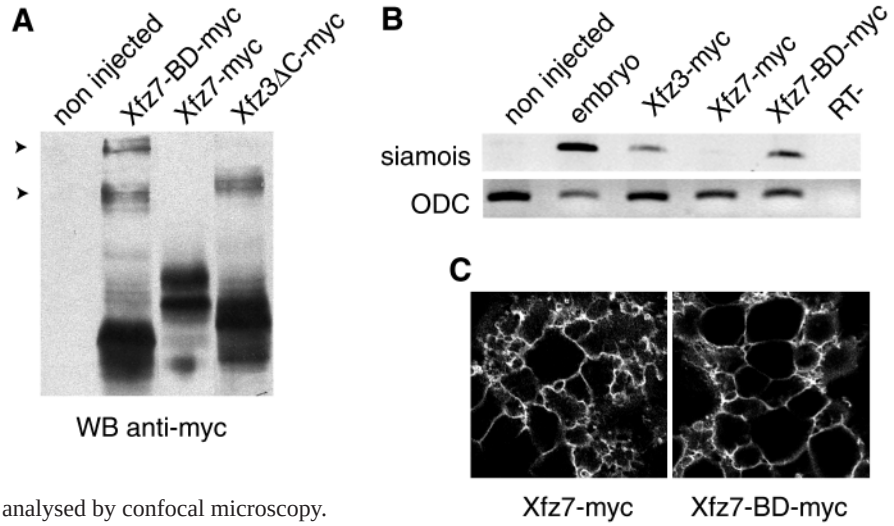

Fig. 8. Oligomerization of Xfz7 is sufficient to activate the Wnt/β-catenin pathway in *Xenopus* embryos. (A) Embryos were injected with mRNA encoding Xfz7-BD-myc, Xfz7-myc and Xfz3ΔC-myc. Protein extracts from injected embryos were separated by SDS-PAGE and immunoblotted with the anti-myc antibody. Xfz7-BD-myc dimer and oligomer are detected (arrowhead). (B) RT-PCR analysis of the Wnt/β-catenin target gene *siamois* in animal caps after overexpression of different frizzled constructs as indicated. RT–: control without reverse transcriptase. ODC is a loading control. Analysis was performed at the early gastrula stage. (C) Localization of Xfz7-BD at the plasma membrane. Immunocytochemistry with the anti-myc antibody, performed on blastula animal caps isolated from Xfz7-myc or Xfz7-BD-myc injected embryos. The subcellular localization was analysed by confocal microscopy.

molecular forms (Fig. 8A), and is correctly expressed at the plasma membrane (Fig. 8B). Expression of Xfz7-BD in animal cap cells induces *siamois* expression (Fig. 8C) at a similar level to that induced by Xfz3. Induced dimerization of Xfz7 is therefore sufficient to activate the Wnt/β-catenin pathway.

Discussion

In this study we have shown that Xfz3, but not Xfz7, is observed on western blot as dimers and/or oligomers when overexpressed in *Xenopus* embryos. Co-immunoprecipitation experiments using differentially epitope-tagged receptors further show the existence of Xfz3 dimers. Although the structural requirements for GPCR dimerization are as yet unknown, several dimerization interfaces have been proposed. These are the extracellular N-terminal domain for the glutamate and calcium-sensing receptors (Bai et al., 1998; Romano et al., 1996; Romano et al., 2001), the intracellular third loop and the sixth transmembrane domain for the β-adrenergic and dopamine receptors (George et al., 1998; Hebert et al., 1996), and the C-tail for the GABA-B and δ-opioid receptors (Cvejic and Devi, 1997; Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Given the ability of the tagged truncated mutant Xfz3ΔC protein to form homodimers and oligomers, the C-tail is clearly not required in the case of Xfz3. Rather, our results suggest that the N-terminal extracellular domain of Xfz3 may play a role in receptor dimerization. In co-immunoprecipitation experiments, two truncated soluble forms of Xfz3 (extra3-flag and extra3-myc) bearing different epitope tags interacted. Using the chimeric protein extra3-R1, composed of the extracelluar domain of Xfz3 fused to the transmembrane and cytoplasmic domains of FGFR-1, we showed that a membrane-bound form of the extracellular N-terminal part of Xfz3 is capable of forming dimeric complexes. Our results are consistent with the fact that crystal structural analyses of CRD, both from secreted frizzled-related protein 3 (sFRP-3) and from mouse frizzled 8 (mFz8), have revealed that CRDs exhibit a putative conserved dimer interface (Dann et al., 2001).

The sensitivity of the dimeric Xfz3 receptor to reducing agents indicates that disulfide bonds are important for Xfz3 dimer formation and/or stabilization. The β-mercaptoethanol treatment can either disrupt intramolecular disulfide bonds and consequently affect conformation of the receptor in a manner that impedes dimerization, or it can disrupt intermolecular disulfide bonds directly responsible for dimer formation. If intermolecular disulfide linkages are implicated in Xfz3 dimerization, none of the ten cysteines located in the CRD is likely to be involved in these interactions as these conserved cysteines have been shown to be engaged in pairwise intramolecular bonds in the secreted frizzled-related proteins sFRP-1 and sFRP-3, as well as in the frizzled module recently determined in rat Ror1 receptor tyrosine kinase (Chong et al., 2002; Roszmusz et al., 2001). However, cysteines outside the CRD could be involved in intermolecular disulfide bonds implicated in Xfz3 dimerization. Several cysteines located in the first and second extracellular loops are conserved among the frizzled proteins and, interestingly, two conserved cysteines, located on these same loops in the m3 muscarinic receptors have been identified as key residues for covalent dimer formation by site-directed mutagenesis (Zeng and Wess, 1999).

Although dimerization has been shown for several GPCRs, the functional roles of such a process are currently unclear. The GABA-B receptors associate in the endoplasmic reticulum as heterodimers and are targeted to the plasma membrane as preformed dimers, independent of agonist regulation (Jones et al., 1998; White et al., 1998). Dimerization of these receptors provides a mechanism to control the efficient delivery of active GPCRs to the cell surface. The β-adrenergic receptor, however, undergoes ligand-dependent dimerization and activation, suggesting that dimerization favors receptor/G-protein coupling efficiency (Angers et al., 2000; Hebert and Bouvier, 1998; Hebert et al., 1996). In the case of frizzled receptors, our results strongly suggest that dimerization plays a role in transducing the signal through the Wnt/β-catenin pathway. When overexpressed in blastula animal cap cells, Xfz3 exists as a dimer and leads to activation of the Wnt/β-catenin pathway, whereas Xfz7 remains monomeric and does not activate the Wnt/β-catenin pathway. Moreover, the addition of a heterologous dimerization domain to Xfz7 is sufficient to activate the Wnt/β-catenin pathway in the animal cap assay.

Activation of this pathway can also be obtained when Xfz7 is co-expressed with an appropriate Wnt ligand, suggesting that the lack of activity of Xfz7 in Wnt/β-catenin signaling in the animal cap cells may be due to the absence of a specific ligand (Umbhauer et al., 2000a).

Although it is tempting to speculate that frizzled dimerization is induced by the Wnt ligand, we have failed to show any reproducible effect of Wnt expression on the frizzled dimer/monomer ratio using western blot or co-immunoprecipitation studies (data not shown). In blastula animal cap cells, the N-terminal extracellular domain of Xfz3 was not sufficient to inhibit Xfz3 dimerization and activation of the Wnt/β-catenin pathway in response to the full-length receptor Xfz3. These results suggest that activation of the Wnt/β-catenin pathway and formation of Xfz3 dimers are both ligand independent, but they do not exclude a different mechanism for the receptor Xfz7. The question of whether ligands affect GPCR oligomerization is currently largely debated. Agonist treatment of several GPCRs has variably been reported to increase, decrease or have no effect on the oligomeric complexes. This may reflect the specific behavior of each receptor or it may be a consequence of using different experimental approaches. For example, immunoprecipitation experiments have suggested that agonists favor monomer formation of the δ-opioid receptor (Cvejic and Devi, 1997), whereas the use of both bioluminescence resonance energy transfer (BRET) and time-resolved fluorescence resonance energy transfer (FRET) approaches does not reveal any effect of agonist or antagonist on this receptor at the cell surface (McVey et al., 2001).

In addition to the canonical Wnt/β-catenin pathway, Wnt and frizzled have been shown to transduce signals through at least two other pathways (Kuhl et al., 2000b; Mlodzik, 1999). Wnt5A and rat frizzled 2 trigger intracellular calcium release (Slusarski et al., 1997) and activate protein kinase C and calmodulin kinase II (CamKII) (Kuhl et al., 2000a; Sheldahl et al., 1999) in a heterotrimeric G-protein-dependent manner. In *Drosophila*, the planar cell polarity pathway (PCP) identified downstream of DFz1 involves the cytoplasmic protein Dsh, the small GTPase RhoA and a Jun N-terminal kinase cascade. A similar PCP pathway might be implicated in the control of gastrulation movements in *Xenopu*s (Wallingford et al., 2000; Yamanaka et al., 2002). Dorsal overexpression of Xfz7 or Xwnt11 perturbs convergence extension movements, which can be rescued by co-injection of a dominant-negative form of the small GTPase (Djiane et al., 2000; Medina et al., 2000; Tada and Smith, 2000). Our results suggest that dimerization of frizzled receptors might not be required for the activation of the PCP pathway, as overexpression of Xfz7 as a monomer is sufficient to alter gastrulation movements. Because several frizzleds seem to be bifunctional receptors capable of transducing signals via two biochemically distinct pathways, dimerization could enable the discrimination between the alternate pathways. Therefore, dimerization of frizzled is emerging as a possible mechanism for transduction specificity.

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