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A role for frizzled 3 in neural crest development

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SUMMARY

Wnts are a large family of secreted molecules implicated in numerous developmental processes. Frizzled proteins are likely receptors for Wnts and are required for Wnt signaling in invertebrates. A large number of vertebrate frizzled genes have also been identified, but their roles in mediating specific responses to endogenous Wnts have not been well defined. Using a functional assay in *Xenopus*, we have performed a large screen to identify potential interactions between Wnts and frizzleds. We find that signaling by Xwnt1, but not other Wnts, can be specifically enhanced by frizzled 3 (Xfz3). As both Xfz3 and Xwnt1 are highly localized to dorsal neural tissues that give rise to neural crest, we examined whether Xfz3 mediates Xwnt1 signaling in the formation of neural crest. Xfz3 specifically induces neural crest in ectodermal explants and in embryos, similar to Xwnt1, and at lower levels of expression, synergizes with Xwnt1 in neural crest induction. Furthermore, loss of Xfz3 function, either by depletion with a Xfz3-directed morpholino antisense oligonucleotide or by expression of an inhibitory form of Xfz3 (Nfz3), prevents Xwnt1-dependent neural crest induction in ectodermal explants and blocks neural crest formation in whole embryos. These results show that Xfz3 is required for Xwnt1 signaling in the formation of the neural crest in the developing vertebrate embryo.

Key words: *Xenopus*, Xwnt, Frizzled, Neural crest, Xfz3, Slug, Vertebrate embryo, Melanocyte

INTRODUCTION

More than 20 Wnt genes have been identified to date and roles for many of these extracellular ligands have been defined in the control of embryonic patterning, organogenesis, cell growth and axonal remodeling (Bejsovec, 1999; Cadigan and Nusse, 1997; Gradl et al., 1999; Miller et al., 1999; Peifer and Polakis, 2000; Salinas, 1999). Frizzleds, which have more recently been identified as receptors for Wnts in both vertebrates and invertebrates, comprise an equally large family (Bhanot et al., 1996; Vinson et al., 1989; Wang et al., 1996). However, for most of these receptors, the endogenous ligand has not been identified. Furthermore, the roles of these multiple frizzleds in development have only been elucidated for a small number, primarily in invertebrates. In *Drosophila*, for example, frizzled (fz) and frizzled 2 (fz2) are both required in the embryo for wingless (wg) signaling (Chen and Struhl, 1999; Bhanot et al., 1999; Bhat, 1998; Kennerdell and Carthew, 1998); fz is also required for proper orientation of hairs in the dorsal epidermis of the adult as well as for establishing the chirality of photoreceptor organization in the eye (Mlodzik, 1999), although in these settings it is not known whether a Wnt is the relevant ligand. In *C. elegans*, frizzleds regulate cell fate, asymmetric cell divisions and spindle pole orientation (Thorpe et al., 2000). Much less is known about the roles of frizzleds in vertebrate development. In *Xenopus*, Xfz7 is required maternally for dorsal axis development and for response to ectopic Xwnt8b (Sumanas et al., 2000), while Xfz8, which is expressed in the Spemann organizer, appears to play a role in organizing cell movements during gastrulation (Deardorff et al., 1998). In zebrafish, fzA, which is closely related to Xfz8, has been proposed to play a role in dorsoventral patterning (Nasevicius et al., 1998). It remains unclear, however, which endogenous ligands activate these or any other vertebrate frizzleds.

Frizzled proteins consist of an extracellular N terminus containing a cysteine-rich domain (CRD) believed to be important for ligand binding, seven hydrophobic domains proposed to be transmembrane segments, and a C-terminal tail believed to be important for cytoplasmic signaling (Vinson et al., 1989). Wg protein has been shown to bind to the surface of Schneider cells that express frizzleds, and a CRD with a GPI anchor also recruits wg to the cell surface (Bhanot et al., 1996). Rat Fz1 expressed in *Xenopus* can also recruit Myc tagged Xwnt8 to the surface of animal cap cells (Yang-Snyder et al., 1996). Although Wnt proteins have been notoriously difficult to purify in an active form, Xwnt8 in conditioned medium, similar to wg, has been shown to bind to the CRD of Fz2 and vertebrate Fz4, Fz5, Fz7 and Fz8 (Hsieh et al., 1999). Importantly, ectopic expression of frizzleds in cells that do not normally respond to Wnts confers Wnt-dependent signal transduction, as assessed by accumulation of armadillo/β-catenin protein (Bhanot et al., 1996). These observations have provided compelling evidence

that frizzled proteins are functional Wnt receptors, although the transmembrane proteins encoded by arrow/LRP-5 and LRP-6 (LDL receptor-like proteins) may interact with frizzleds and serve as co-receptors for Wnts (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000).

Because of the large number of Wnts and frizzleds expressed in vertebrate embryos (Wang et al., 1996), it is important to define which Wnts interact with specific frizzleds. Straightforward biochemical analysis, particularly binding assays, have in general been limited by the difficulty in obtaining purified soluble Wnts, except in limited cases. An alternative and informative approach has been to examine synergy between ligand and receptor pairs. Using this approach, He et al. have demonstrated that Xwnt5a, when ectopically expressed with human FZ5 (FZD5 – Human Gene Nomenclature Database; originally named hfz5), is able to activate the canonical Wnt pathway and cause duplication of the dorsal axis in *Xenopus* embryos, despite the inability of Xwnt5a to do so on its own (He et al., 1997), which suggests a functional interaction between *Xenopus* Xwnt5a and human FZ5. Similarly, *Xenopus* Xwnt8 and rat Fz1 have been shown to synergize in the activation of dorsal specific gene expression (Yang-Snyder et al., 1996) and Xfz8 has been shown to synergize with Xwnt8 in dorsal axis duplication assays (Deardorff et al., 1998). Recently, two groups have also demonstrated synergy between Xwnt8b and Xfz7 in the dorsal axis duplication assay in *Xenopus* (Medina et al., 2000; Sumanas et al., 2000).

Using this approach, we have examined eight Wnts and five frizzleds for potential interactions in the dorsal axis induction assay in *Xenopus*. This screen identified a remarkably strong interaction between Xwnt1 and Xfz3, while none of the other Wnts tested showed significant interaction with Xfz3. As Xwnt1 and Xfz3 are both expressed in the dorsal neural tube in *Xenopus*, zebrafish and mouse (Fig. 2a; Borello et al., 1999; Dorsky et al., 1998; Shackleford and Varmus, 1987; Shi et al., 1998; Wilkinson et al., 1987; Wolda et al., 1993) and Xwnt1 has been implicated in the regulation of neural crest development (Dorsky et al., 1998; Ikeya et al., 1997; Saint-Jeannet et al., 1997), we also examined whether Xfz3 may also play a role in neural crest development. We find that Xfz3 is able to induce neural crest in embryos and in explants and that inhibition or loss of Xfz3 blocks endogenous neural crest formation. These observations suggest that endogenous Xwnt1 and Xfz3 act as a ligand-receptor pair in the induction of neural crest.

MATERIALS AND METHODS

cDNA cloning and constructs

In several screens of maternal and stage 15 cDNA libraries to identify *Xenopus* frizzled homologs, we isolated cDNAs encoding the full open-reading frames of Xfz2 (Deardorff and Klein, 1999), Xfz3 (Shi et al., 1998), Xfz7(Gradl et al., 1999) and Xfz8(Deardorff et al., 1998). The open reading frame of each frizzled cDNA was amplified by PCR, adding *Eco*RI and *Xho*I sites to the 5′ and 3′ ends respectively; these were then subcloned into pCS2+ (Turner and Weintraub, 1994) and verified by sequencing. The N-terminal domain of Xfz3 (Nxfz3, amino acids 1-202), which comprises the putative ligand-binding CRD, was subcloned into pCS2 and pCS2MT (which adds a C-terminal 6X Myc tag) using *Bam*HI and *Cla*I sites added by PCR. Xwnt1 (Noordermeer et al., 1989) was recloned by PCR from a stage 30 head library cDNA (gift of Richard Harland) into pCS2. Mouse Wnt1 (Gavin et al., 1990) and mouse Fzd3 (Wang et al., 1996) were also subcloned into pCS2.

In vitro transcription, microinjection and explants

Capped synthetic RNAs were generated using an SP6 mMessage mMachine kit from Ambion (Austin, TX). RNA in a volume of 10 nl was injected into embryos as described (Deardorff et al., 1998). For synergy assays, each RNA preparation was tested independently in a dose-response analysis to identify concentrations that were ineffective alone in dorsal axis duplication or neural crest induction assays. pCS2/nβgal was used to generate lineage tracer RNA. For morpholino oligo injections (Heasman et al., 2000), a Xfz3 antisense oligo with the sequence 5′-CGCAAAGCCACATGCACCTCTTGAA-3′ was purchased from Gene Tools (Corvallis, OR) and was dissolved in DEPC-treated water. 5 nl of oligo (0.4 ng/nl) was injected into one dorsolateral animal blastomere at the 32-cell stage together with mRNA encoding β-galactosidase. Embryos were cultured until stage 18 and then fixed and stained for β-galactosidase activity and *Xslug* expression as described below.

Fertilization and embryo culture were performed as described previously (Deardorff et al., 1998). The neural crest induction assay in animal pole explants was performed as described previously (Saint-Jeannet et al., 1997), except that chordin was used instead of noggin; briefly, embryos were injected at the 1-2 cell stage in the animal pole with mRNA for chordin alone or with Xwnt1, frizzleds or frizzled deletion constructs in combinations and at concentrations indicated in the figure legends. At stage 9, animal pole explants were removed using a Gastromaster, were cultured as described previously (Deardorff et al., 1998) and were then harvested at the stages indicated in the figure legends.

RT-PCR

RT-PCR methods and primers for EF-1α, muscle actin and NCAM are described elsewhere (Deardorff et al., 1998). Primers for *Xslug*, *Xsnail*, *Xtwist* (LaBonne and Bronner-Fraser, 1998) and *ADAM-13* (Alfandari et al., 1997) were as reported. In addition, primers for *Xpax3*, *Xfz3* and *Xwnt1* were as follows (in each case 25 cycles of amplification were used and conditions were otherwise as described (Deardorff et al., 1998): Pax3, U-ACCACATTCACTGCAGAGC D-AACCACACTTGAACTCGCG; Xfz3, U-TAACAATCATCCTG-CTCGC D-TTGTACCCAAGTTGTCTCC; Xwnt1, U-TGCTGTTT-CTGCCTTGGGTG D-GCGTCGGTTCCTAAAATGCC

In situ hybridization, histology and lineage tracing

Whole-mount in situ hybridization was performed as described (Deardorff et al., 1998). Probes detected *Xfz3*, *Xslug* (Mayor et al., 1995) and *Xwnt1* (Wolda et al., 1993). Lineage tracing was performed by co-injecting β-galactosidase RNA and staining the embryos using Red-Gal (Research Organics) as a substrate.

Immunoblotting

Xenopus embryos were injected at the animal pole at the one cell stage with Xfz3 mRNA and varying doses of Xfz3 morpholino antisense oligonucleotide (as indicated in Fig. 8), developed to stage 10 and lysed in embryo lysis buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 10% glycerol, 1 mM DTT, 2 mM sodium vanadate, 25 mM NaF, 1% Nonidet P-40, and protease inhibitor cocktail for mammalian cells (Sigma)). Protein extracts were analyzed by western blot using α-Xfz3 monoclonal antibodies raised against GST-Xfz3 C terminus, amino acids 499-664 (Tan et al., 2001).

RESULTS

Assay for synergy between Wnts and frizzleds in dorsal axis duplication

To identify specific, functional interactions between Wnts and frizzleds, we tested for synergy in a previously described assay for dorsal axis induction assay in *Xenopus* embryos (Deardorff

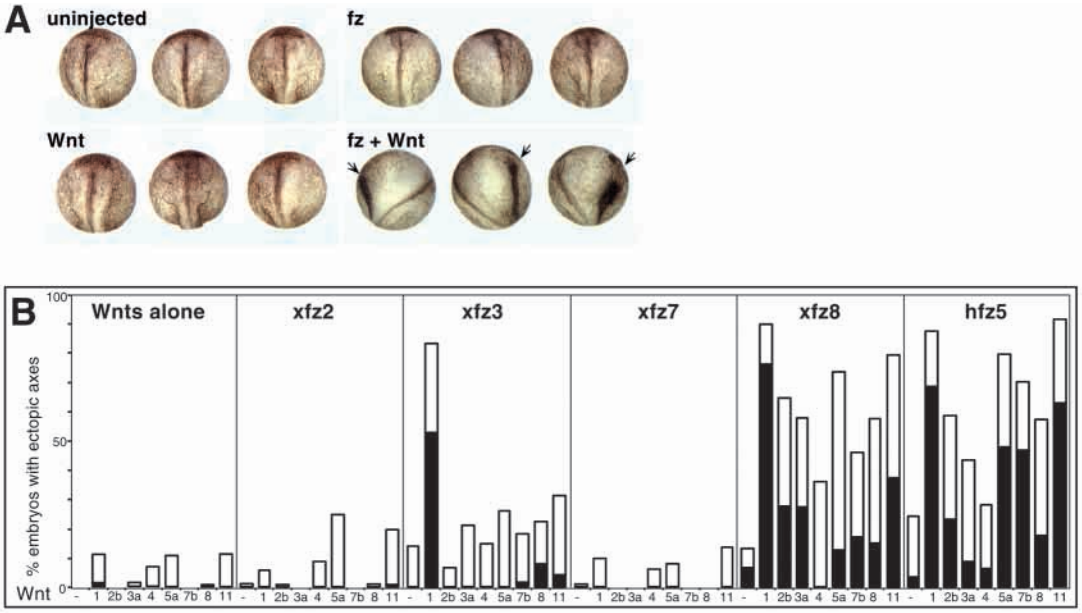

Fig. 1. Xwnt1 and Xfz3 synergize in the dorsal axis induction assay. (A) *Xenopus* ectopic axis assay showing synergy between Wnts and frizzleds. RNAs encoding frizzleds (Xfz) and Wnts were injected at low concentrations either alone or in combination into a ventral blastomere of a four- to eight-cell embryo. Secondary axes (indicated by arrows) were scored at the late neurula stage. (B) Summary of screen to identify synergistic Wnt/frizzled combinations. Percent of embryos with ectopic axes is plotted with respect to RNAs injected. The black represents complete axes with cement gland and at least one eye at stage 30. The white represents partial axes. *n*=61 per combination. Doses of RNA used: Xfz2 (1 ng), Xfz3 (100 pg), Xfz7 (500 pg), Xfz8 (20 pg), mouse Wnt1 (1 pg), Xwnt2b (2.5 pg), Xwnt3A (0.5 pg), Xwnt4 (1 ng), Xwnt5A (200 pg), Xwnt7b (0.1 pg), Xwnt8 (25 pg) and Xwnt11 (500 pg). Synergy in the axis duplication assay was similar for *Xenopus* and mouse Xwnt1 and frizzled 3 (data not shown).

et al., 1998; He et al., 1997; Medina et al., 2000; Sumanas et al., 2000; Yang-Snyder et al., 1996). This assay uses doses of Wnts and frizzleds that alone induce few or no ectopic axes. When co-expressed, synergistic Wnts and frizzleds induce a high percentage of ectopic axes (arrows in Fig. 1A), suggesting potential ligand-receptor relationships. In this assay, we tested four *Xenopus* frizzleds (for a review see Gradl et al., 1999) and human FZ5 (Wang et al., 1996) with eight *Xenopus* Wnts (Gradl et al., 1999) as indicated in Fig. 1B. Of the 40 combinations tested, specificity between Xfz3 and Xwnt1 stood out, with co-expression resulting in a high percentage (>80%) of ectopic axes. Other Wnts failed to demonstrate strong synergy with Xfz3. Xfz8 and human FZ5 appear to synergize with many Wnts, indicating apparently reduced specificity. By contrast, Xfz2 and Xfz7 did not show marked synergy with any of the Wnts tested (Xwnt8b was not tested; see Medina et al., 2000; Sumanas et al., 2000).

Xfz3, like Xwnt1, induces neural crest

The strong synergy between Xwnt1 and Xfz3 suggests that they may function together in vivo. Indeed, Xwnt1 and Xfz3 mRNAs are expressed in overlapping domains in the dorsal neural tube in *Xenopus* (Shi et al., 1998; Wolda et al., 1993; and data not shown) and mouse (Borello et al., 1999; Shackleford and Varmus, 1987; Wilkinson et al., 1987), consistent with a role as an endogenous ligand-receptor pair in dorsal neural tissue. In this location, Xwnt1 has been shown to play a role in the developing neural crest in mouse (Ikeya et al., 1997), zebrafish (Dorsky et al., 1998) and *Xenopus* (Saint-Jeannet et al., 1997). In *Xenopus*, however, neural crest induction begins in the early neurula stage and continues after closure of the neural tube (Mayor et al., 1995). Consistent with an early role in neural crest formation, Xfz3 and Xwnt1 are also expressed in overlapping domains in the neural plate before closure of the neural tube (Shi et al., 1998; Wolda et al., 1993). In agreement with Shi et al., whole-mount in situ analysis of stage 18 neurulae shows expression of Xfz3 in the neural plate, highest in the anterior but detectable in the trunk as well **(**Fig. 2A). Xwnt1 is expressed in a longitudinal stripe that is most intense in the presumptive hindbrain and gradually declines posteriorly, overlapping the expression of Xfz3, as well as in a mediolateral stripe that corresponds to the future midbrain-hindbrain boundary. Although Xwnt1 and Xfz3 are expressed at earlier stages, the levels of expression are lower and difficult to detect by whole-mount in situ analysis. However, Xfz3 mRNA can be detected by RNase protection and RT-PCR throughout early development, with an increase beginning in the early neurula (Fig. 2B; Shi et al., 1998; Tan et al., 2001). In parallel with the increase in Xfz3 expression, Xwnt1 expression increases markedly by stage 14, coincident with the expression of dorsal neural and neural crest markers such as *Xpax3* and *Xslug* (Fig. 2B; Mayor et al., 1995; Sadaghiani and Thiebaud, 1987; Wolda et al., 1993). Thus Xwnt1 and Xfz3 are co-expressed in neural tissues from the neurula stage through tadpole stages, and may therefore function as a ligand-receptor pair in the formation or patterning of dorsal neural tissues such as neural crest.

Wnts have been shown to cooperate with neuralizing signals to induce robust expression of neural crest markers in *Xenopus* (Bang et al., 1999; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997). As Xfz3 synergizes with Xwnt1 and is expressed in tissues that

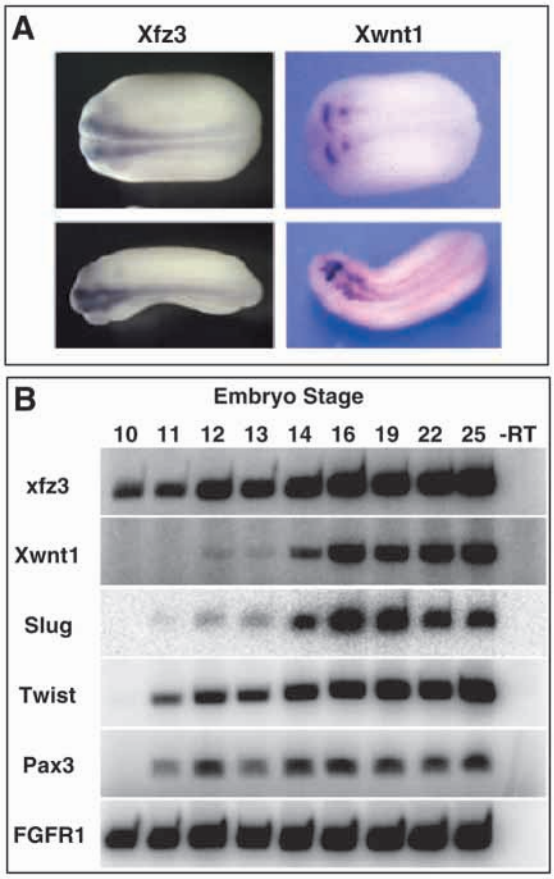

Fig. 2. Xfz3 and Xwnt1 are co-expressed in developing neural tissues. (A) Whole-mount in situ analysis of Xfz3 and Xwnt1 in neurula stage embryos (top, stage 18) and early tadpoles (bottom, stage 25). (B) Embryos were harvested at stages indicated and expression of dorsal neural markers was assessed by RT-PCR. Fgf receptor (FGFR1) was used as a loading control.

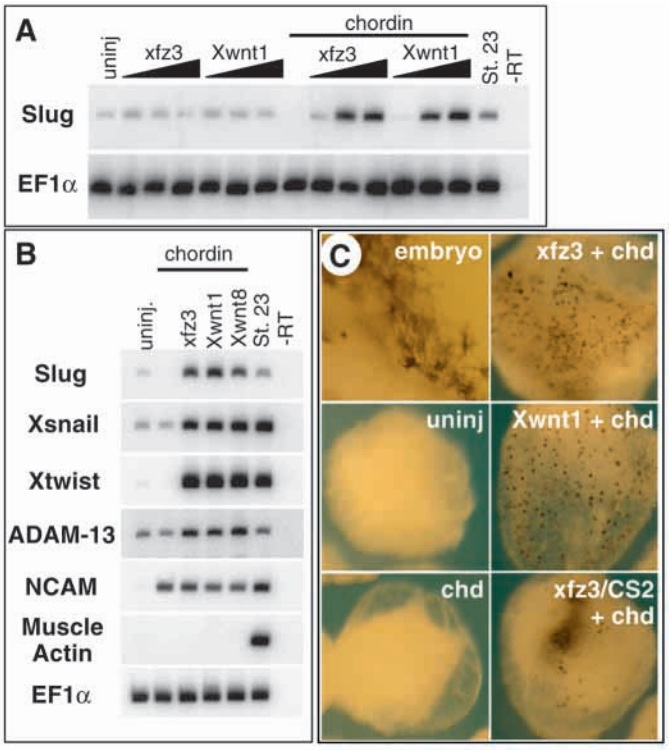

Fig. 3. Xfz3 induces neural crest in animal caps. (A) Xfz3 induces neural crest in a dose-dependent manner. RT-PCR was performed on animal caps ectopically expressing Xfz3 (10 pg, 100 pg, 1 ng) or Xwnt1 (1 pg, 10 pg, 100 pg), either alone or with chordin (1 ng). Stage 23 embryos were used as a positive control. -RT, without reverse transcriptase; EF1α is a loading control. (B) Xfz3 induces the same neural crest markers as Xwnt1 and Xwnt8, shown by RT-PCR analysis of animal caps expressing chordin (1 ng) alone or with Xfz3 (1 ng), Xwnt1 (0.1 ng) or Xwnt8 (0.1 ng). (C) Xfz3 induces migratory melanocytes in animal caps. Albino eggs were fertilized with pigmented male sperm and injected with RNAs for chordin (1 ng) alone or with Xfz3 (100 pg), Xwnt1 (10 pg) or Xfz3/CS2 (50 pg) as indicated. Punctate pigmented spots are migratory melanocytes. 'uninj' indicates animal cap from uninjected embryo (A-C). 'embryo' in C (upper right) indicates close-up view of melanocytes in uninjected embryo.

develop into neural crest, we tested whether Xfz3 can induce neural crest in ectodermal explants. We find that Xfz3, similar to Xwnt1, induces *Xslug* in a dose-dependent manner in animal cap explants that have been neuralized by expression of chordin, but not in non-neuralized explants (Fig. 3A). Furthermore, Xfz3, like Xwnt1, also induces the neural crest markers *Xsnail* (Essex et al., 1993), *Xtwist* (Hopwood et al., 1989) and *ADAM-13* (Alfandari et al., 1997; Fig. 3B). This induction of neural crest by Xfz3 is not a result of secondary induction by paraxial mesoderm as indicated by the absence of muscle actin in these explants (Fig. 3B). Interestingly, Xfz3 does not reduce the expression of the anterior neural marker Otx2 or induce expression of the posterior neural marker hoxB9 (data not shown), indicating that Xfz3, in contrast to Xwnt1, Xwnt3a, Xwnt8 and Xwnt7b (McGrew, 1995; Fredieu, 1997; Chang, 1998), does not posteriorize the anterior neural tissue induced in these assays.

To ensure that differentiated neural crest cells (LeDouarin, 1982) form in these assays, we cultured explants until stage 40, when neural crest-derived melanocytes are easily detectable in sibling tadpoles. Albino eggs fertilized with wild-type sperm were used for these assays to facilitate visualization of melanocytes, as maternally derived pigment is absent from these explants. As shown in Fig. 3C, both Xfz3 RNA (93%, *n*=60) and Xfz3 expressed from a plasmid (Xfz3/CS2; 85%, *n*=53) induce differentiated melanocytes in neuralized explants while caps from uninjected embryos (0%, *n*=73) or those expressing chordin alone (4%, *n*=71) have few melanocytes. Xwnt1 (96%, *n*=73) also induced the formation of melanocytes, consistent with previous work in zebrafish (Dorsky et al., 1998) as well as observations in the mouse that Xwnt1 and Xwnt3a are required for melanocyte development (Ikeya et al., 1997; Dunn et al., 2000). These findings indicate that both Xfz3 and Xwnt1 induce differentiated neural crest and suggest that Xfz3 and Xwnt1 function in the same signaling pathway in neural crest formation.

In order to determine whether Xfz3 induces neural crest at the same time as endogenous neural crest formation, animal caps expressing chordin and Xfz3 were harvested at several intervals from stage 12 (late gastrula) until stage 25 (tadpole). In parallel, caps expressing chordin and Xwnt1, as well as whole embryos, were also harvested and *Xslug* expression was

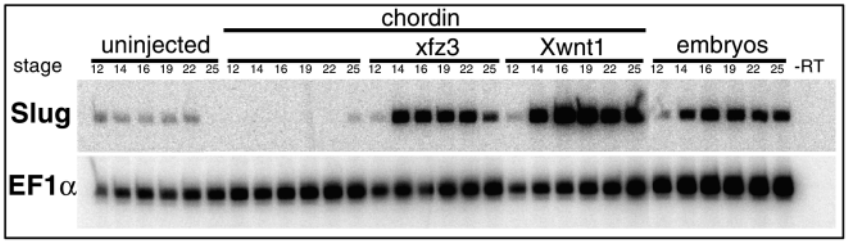

Fig. 4. Xfz3 and Xwnt1 induce *Xslug* at similar times in early neurula stage explants, in parallel with onset of Xslug expression in whole embryos. Animal caps expressing chordin, chordin plus Xfz3, or chordin plus Xwnt1, as well as control animal caps and sibling embryos, were harvested at the stages indicated and processed for RT-PCR analysis as in Fig. 3.

measured as above. As shown in Fig. 4, *Xslug* expression is strongly induced by stage 14, similar to the effect of Xwnt1 and similar to *Xslug* expression in whole embryos. Thus, the timing of *Xslug* induction by Xfz3 is similar to both Xwnt1 and endogenous *Xslug* expression.

Xfz3 synergizes with Xwnt1 in neural crest induction

The above data are consistent with the suggestion that Xfz3 functions as a receptor for Xwnt1 in neural crest development. While it has been difficult to obtain soluble Xwnt1 protein for direct binding assays, we wished to test this possibility further. Using a synergy assay for neural crest similar to the axis duplication assays (Fig. 1), we used doses of Xwnt and Xfz RNA that induce low levels of neural crest when expressed alone in neuralized animal caps (Fig. 5A). When Xwnt1 and Xfz3 were co-expressed, a marked increase was observed in the induction of *Xslug*. This synergy was not observed with co-expression of Xwnt8 and Xfz3, as indicated by quantitation below each lane.

An inhibitory form of Xfz3 blocks neural crest induction by Xwnt1

In order to interfere with Xwnt1/Xfz3 interactions, we generated an inhibitory form of Xfz3 (Nxfz3) and tested its function in the animal cap model of neural crest formation (Fig. 5B). Nxfz3 encodes the N-terminal cysteine-rich domain (CRD), which in other frizzleds binds Wnts and has been shown to inhibit Wnt signaling (Bhanot et al., 1996; Deardorff et al., 1998; Gradl et al., 1999; He et al., 1997; Hsieh et al., 1999). Nxfz3 inhibits the Xwnt1-dependent induction of *Xslug* in a dose-dependent manner (Fig. 5B), suggesting that Xfz3 is required for Xwnt1-mediated neural crest induction in these explants. Furthermore, inhibition by Nxfz3 is overcome by increasing doses of Xwnt1 (Fig. 5B), suggesting that Nxfz3 competitively inhibits Xwnt1 binding to endogenous Xfz3. The synergy observed between Xwnt1 and Xfz3 and the competition between Xwnt1 and Nxfz3 provide additional support for the proposal that Xwnt1 and Xfz3 function in a ligand-receptor relationship.

The observation that overexpression of Xfz3 leads to neural crest induction raises the question of whether Xfz3 functions constitutively or is dependent on an endogenous ligand (such as Xwnt1). To address this question, Xfz3 was co-expressed with Nxfz3 in neuralized animal caps. Nfxz3 prevented neural crest induction by full-length Xfz3, suggesting that the activity of Xfz3 in this assay is dependent on an endogenous ligand

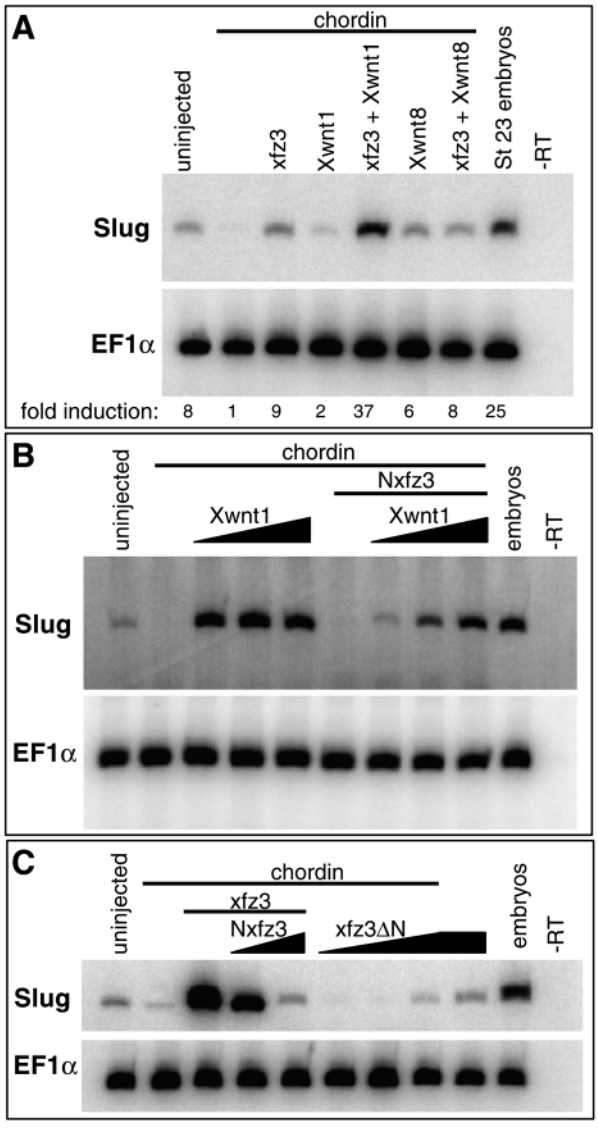

Fig. 5. Interaction between Xwnt1 and Xfz3 in neural crest induction. (A) Xwnt1 synergizes with Xfz3 to induce *Xslug*. RT-PCR analysis of *Xslug* expression in animal caps expressing chordin (1 ng) alone, with Xfz3 (10 pg), Xwnt1 (1 pg) or Xwnt8 (1 pg) or combinations thereof. Shown below each lane are the *Xslug* expression levels, normalized to EF1α, relative to the chordin alone sample (defined as 1). (B) An inhibitory form of Xfz3 encoding the CRD (Nfz3) inhibits neural crest formation and this is overcome by increasing Xwnt1. RT-PCR analysis of *Xslug* expression in animal caps expressing chordin (1 ng) alone, with Xwnt1 (10 pg, 30 pg, 100 pg), with Nfz3 (4 ng) or combinations thereof. Controls are as in Fig. 3. (C) Activity of Xfz3 in neural crest induction requires the CRD (putative ligand binding domain). Co-expression of Xfz3 with Nxfz3 blocks induction of *Xslug* by Xfz3 and a construct lacking the CRD (Xfz3ΔN) is not active in neural crest induction. (All samples were harvested at stage 23.)

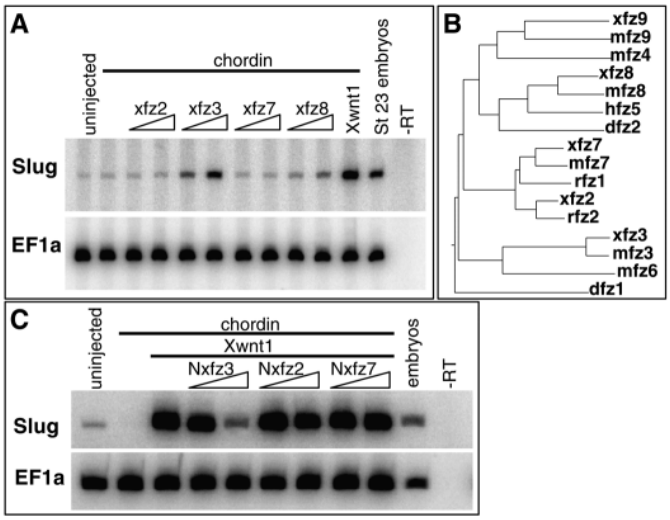

Fig. 6. (A) Xfz3 specifically induces neural crest. RT-PCR analysis of *Xslug* expression is shown for animal caps expressing chordin (1 ng) alone or with Xfz2 (0.1 ng, 1 ng), Xfz3 (0.1 ng, 1 ng), Xfz7 (0.1 ng, 1 ng), Xfz8 (0.1 ng, 1 ng) and Xwnt1 (0.1 ng). (B) Dendrogram analysis of *Drosophila*, mouse and *Xenopus* frizzleds. (C) Nxfz3, but not Nxfz2 or Nxfz7, inhibits Xwnt1 induction of *Xslug*. RT-PCR analysis of *Xslug* expression in animal caps expressing chordin (1 ng) alone, Xwnt1 and Nxfz3, 2 or 7 (0.1 and 1.0 ng).

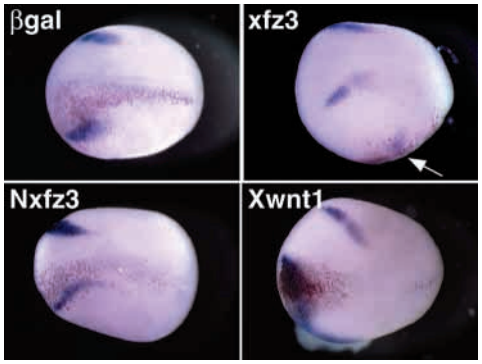

Fig. 7. Analysis of Xfz3 activity in whole embryos. Albino embryos were injected at the two cell stage with RNA into the animal pole region of either the left or right blastomere. RNAs used were Xfz3 (0.75-3.0 ng), Nfz3 (0.75-3.0 ng), Xwnt1 (10 pg) and the lineage tracer β-galactosidase (200 pg). The red nuclear β-galactosidase staining indicates the side injected. The dark purple indicates *Xslug* expression. All panels are dorsal views with anterior to the left; the view of Xfz3 is slightly oblique with the arrow indicating ectopic *Xslug* on the injected ventral lateral side of the embryo.

(Fig. 5C). Furthermore, a Xfz3 construct that lacks the CRD (Xfz3ΔN) has no detectable activity (Fig. 5c), indicating that the CRD is required for Xfz3 function in this assay. These observations are in contrast to Xfz8, which still dorsalizes when co-expressed with a construct encoding only the CRD (Nxfz8) (Deardorff et al., 1998) and shows apparent constitutive activity when the CRD is deleted (data not shown).

Specificity of Xfz3 in neural crest induction

To test whether neural crest induction is a specific activity of Xfz3 or a general attribute of frizzleds, we analyzed Xfz2,

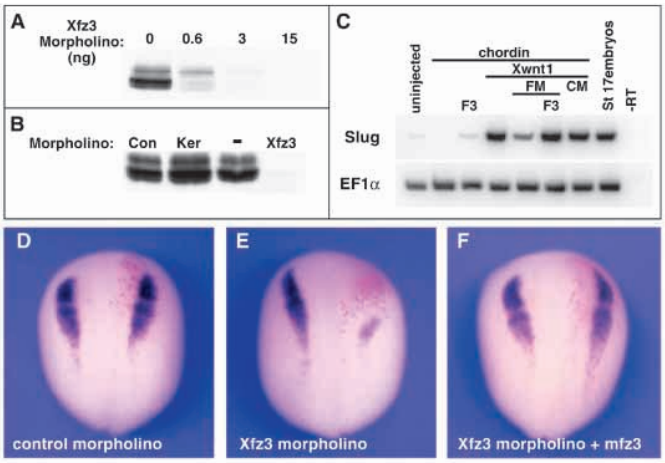

Fig. 8. Xfz3 is required for neural crest formation. (A) Xfz3-directed antisense morpholino oligonucleotide blocks translation of injected Xfz3 mRNA. Xfz3 mRNA was injected into one cell embryos together with Xfz3 morpholino at the doses indicated. Embryos were harvested at stage 10 and analyzed by western blot with Xfz3 antibodies. (B) Xfz3 mRNA was injected together with control (Con), Kermit (Ker) or Xfz3 morpholinos (2 ng each) or with no oligo (-) and stage 10 embryos were western blotted for Xfz3 as above. (C) RT-PCR analysis of *Xslug* expression (as in previous figures) in animal caps expressing chordin and Xwnt1, co-injected with either Xfz3 morpholino (FM; 4 ng) or control morpholino (CM; 4 ng) and harvested at stage 18. Xwnt1-dependent induction of Xslug was rescued by co-injection of an Xfz3 mRNA (F3) lacking the morpholino target sequence; this dose of F3 mRNA (10pg) caused minimal induction of *Xslug* in the absence of Xwnt1. (D-F) 2 ng of the control morpholino (D) or Xfz3 morpholino (E,F) were co-injected with mRNA for nuclear β-galactosidase into a single dorsal-lateral (B2) blastomere at the 32-cell stage. For rescue of Xfz3 depletion, mouse Xfz3 mRNA (0.4 ng) was co-injected with Xfz3 morpholino (F). Embryos were fixed at stage 18 and β-galactosidase activity was measured in situ (magenta) followed by whole-mount in situ hybridization for *Xslug*.

Xfz3, Xfz7 and Xfz8 for induction of neural crest in animal caps. Neural crest induction was observed with Xfz3, but not with Xfz2 or Xfz7 and only weakly with Xfz8, as assessed by *Xslug* induction (Fig. 6A). Thus, Xfz3 is functionally different from the other frizzleds with respect to induction of neural crest. This is supported by the sequence comparison of Xfz3 that places it in a different sequence subfamily from the other three frizzleds tested (see dendrogram in Fig. 6B). Furthermore, the CRD domains of Xfz2 and Xfz7 (Nfz2 and Nfz7) do not inhibit neural crest induction, indicating that Nfz3 is a specific inhibitor of neural crest induction (Fig. 6C), although it remains possible that Nfz3 interacts with other unidentified Wnts in this assay.

Frizzled-3 is required for neural crest formation in whole embryos

So far, our data suggest that Xwnt1 and Xfz3 interact to induce neural crest in neuralized animal caps. To extend this analysis to whole embryos, RNAs expressing Xfz3, Nxfz3 or Xwnt1 were co-injected with a lineage tracer (β-galactosidase; red) into one blastomere of two cell embryos. At neurula stages, embryos were subjected to whole-mount in situ hybridization for expression of *Xslug* (Fig. 7). Consistent with a role for Xfz3

in neural crest induction, we observed increased *Xslug* expression on the injected side in 83% of cases (*n*=81). Furthermore, the Nxfz3-expressing embryos showed a reduction in *Xslug* expression on the injected side in 63% of embryos (*n*=76). As a positive control, we observed that *Xwnt1*-injected embryos showed increased *Xslug* expression in 98% of cases (*n*=43), while β-galactosidase RNA alone had no effect on the symmetric expression of *Xslug* (100% symmetric; *n*=45). Similar to observations in the explant assays (Fig. 3C), later expression of Xfz3 using plasmid injections also resulted in increased expression of *Xslug* in whole embryos, although at a lower frequency than with RNA injections (43%, *n*=79, data not shown).

A more rigorous test of the requirement for Xfz3 in endogenous neural crest formation would be to deplete Xfz3 protein. Thus, we have used a morpholino antisense oligonucleotide (MO) directed against the 5′ untranslated region of Xfz3 to block translation of Xfz3. Fig. 8A shows that the Xfz3 MO completely blocks translation of injected Xfz3 mRNA while the control MO has no effect on Xfz3 levels; although antibodies to Xfz3 are not sensitive enough to detect endogenous Xfz3 protein, it is likely that endogenous Xfz3 translation is also robustly inhibited under these conditions. In the explant assay described above, the Xfz3 MO blocked Xwnt1-dependent induction of *Xslug* (Fig. 8c), while the control MO had no effect; furthermore, Xwnt1-dependent induction of *Xlsug* was rescued by co-injection of a Xfz3 construct lacking the MO target sequence, showing that Xfz3 is required for the response to Xwnt1 in this assay. Note that the dose of this Xfz3 mRNA (10 pg) caused minimal induction of *Xslug* in the absence of Xwnt1. The Xfz3 MO or control MO was then injected together with β-galactosidase mRNA at the 32-cell stage into dorsal-animal hemisphere cells fated to become neural crest (Moody, 1987). Embryos were fixed at late neurula stages (stage 18) and stained for β-galactosidase activity and *Xslug* expression. For samples in which β-galactosidase activity was observed in the neural crest forming region, the Xfz3 MO markedly reduced *Xslug* expression (Fig. 8D; 56%, *n*=86). This effect was rescued by co-injection with mouse Xfz3 (Fig. 8E), which encodes a highly similar Xfz3 open reading frame but does not hybridize to the *Xenopus* Xfz3 MO (only 13% showed reduced slug expression; *n*=32). Mouse Fzd3, when injected alone at this rescuing dose, had no apparent effect on *Xslug* expression. Thus Xfz3 is required for neural crest formation in whole embryos as well as for the response to Xwnt1.

DISCUSSION

In summary, examination of potential interactions between five frizzled genes and eight Wnt genes identified a specific functional interaction between Xwnt1 and Xfz3. This observation leads us to propose a role for Xfz3 as an endogenous receptor for Xwnt1 in the development of the neural crest, an hypothesis that is supported by several observations: (1) Xfz3 and Xwnt1 are both expressed in the dorsal neural tissues that give rise to neural crest; (2) ectopic Xfz3 mimics Xwnt1 in the timing and character of neural crest induction in embryos as well as in neuralized explants; (3) Xfz3 enhances the response to Xwnt1 in neural crest induction; (4) The putative ligand binding domain of Xfz3 (Nxfz3) inhibits Xwnt1-dependent neural crest induction, and this can be overcome by increased levels of Xwnt1, consistent with competitive inhibition (Nxfz3 also blocks neural crest formation in whole embryos which we propose occurs through binding and consequent inhibition of endogenous Xwnt1.); and (5) Depletion of Xfz3 by antisense oligonucleotides shows that Xfz3 is required for endogenous neural crest formation and for Xwnt1-dependent neural crest induction in neuralized explants.

Biochemical analysis of Wnt/frizzled binding available to date (Hsieh et al., 1999) appears to correlate with functional data we have obtained from dorsal axis synergy assays. Thus, Xwnt8 binds to Xfz8 but not Xfz3 (Hsieh et al., 1999), and similarly synergizes with Xfz8 but not with Xfz3 (this work). Clearly it will be essential to correlate functional analysis with direct binding assays for Xwnt1when sufficient levels of active, soluble protein are attainable.

Several other findings also support a specific functional interaction between Xwnt1 and Xfz3. Xfz3 alone does not induce axis duplication (or expression of dorsal mesodermal markers), even when expressed at high levels (M. A. D. and P. S. K., unpublished; Shi et al., 1998; Sheldahl, 1999), despite the presence of several maternal Wnt mRNAs (Gradl et al., 1999). However, Xfz3 greatly enhances the response to ectopic Xwnt1, suggesting that Xfz3 is both specific for Xwnt1 and that its activity in these assays is dependent on ligand activation. Indeed, the induction of neural crest by ectopic Xfz3 is blocked by co-expression of Nfz3, suggesting that this activity depends on the endogenous Xwnt1 ligand in early embryos. Other Xwnts that do not appear to synergize with Xfz3, including Xwnt3a, Xwnt7b and Xwnt8, are also able to induce neural crest in neuralized explants; these ligands may function through a promiscuous Xfz, such as Xfz8 (Fig. 1B), which is present in animal caps (data not shown). Alternatively, these ligands may interact with Xfz9, which is expressed in a pattern similar to Xfz3 in anterior neural tissues in the tadpole (Wheeler and Hoppler, 1999).

Previous work has suggested that overexpression of mouse Xfz3, which is closely related to *Xenopus* Xfz3, activates protein kinase C (PKC) but not expression of the Wnt-dependent gene siamois (*Xsia*) in *Xenopus* (another Wnt-dependent dorsal gene, Xnr3, was weakly activated (Sheldahl et al., 1999)). By contrast, a more recent report showed that overexpression of *Xenopus* Xfz3 can induce the dorsal markers *Xsia* and *Xnr3*, consistent with ectopic activation of the canonical Wnt pathway, although that work did not show dorsal axis induction by Xfz3 (Umbhauer et al., 2000). These apparently conflicting observations may reflect differences in the constructs used (for example mouse versus *Xenopus*) or in the level of overexpression of the respective mRNAs. Consistent with both studies, we found that neither Xfz3 (Fig. 1) nor mouse Fz3 (Fzd3 – Mouse Genome Informatics; data not shown) induces secondary dorsal axes in the absence of Xwnt1. However, Xfz3 in combination with Xwnt1 robustly induces secondary axes, implying that the Xwnt1/Xfz3 interaction activates the canonical Wnt/β-catenin pathway. The ability of frizzleds to function in both canonical and non-canonical pathways has been reported for *Drosophila* fz and fz7, and raises the interesting possibility that the ligand may determine which pathway is activated by a given fz (Bhanot et al., 1999; Bhat, 1998; Chen and Struhl, 1999; Djiane et al.,

2000; Kennerdell and Carthew, 1998; Medina et al., 2000). As the canonical Wnt/β-catenin pathway has been strongly implicated in neural crest formation (Dorsky et al., 1998; Dorsky et al., 2000; Saint-Jeannet et al., 1997), it seems likely that activation of this pathway by Xwnt1/Xfz3 interaction is also responsible for neural crest induction by this putative ligand-receptor pair observed here. However, the possibility that activation of PKC (or another effector) by Xfz3 plays a role in neural crest induction cannot be ruled out.

Work in the mouse has suggested that Wnts are required for the expansion of neural crest progenitors (Ikeya et al., 1997; Dunn et al., 2000), while work in *Xenopus* and zebrafish (Bang et al., 1999; Chang and Hemmati-Brivanlou, 1998; Dorsky et al., 1998; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997) supports a more direct role for Wnt signaling in specification of neural crest lineage. The work presented here, together with our related work on the Xfz3-interacting protein Kermit (Tan et al., 2001), suggests that Xfz3 signaling is required for neural crest formation; however, the generation of neural crest is a dynamic process and it is not yet clear whether Xwnt1/Xfz3 signaling is involved in the earliest steps in neural crest induction or plays a later role in maintenance and/or proliferation of neural crest.

Fz3 expression in *Xenopus* is mostly restricted to the developing CNS, especially the dorsal neural tube, but also including the developing optic and otic vesicles (Shi et al., 1998; data not shown). Thus, while this work identifies a role for Xfz3 in neural crest induction, possible additional roles are not addressed here. Indeed, Xfz3 appears to play a role in the early development of the eye, as overexpression of Xfz3 can lead to ectopic formation of complete eyes and antagonists of Xfz3 function, such as Nfz3, block endogenous eye formation (Rasmussen et al., 2001).

In this work, we have identified a synergistic interaction between Xwnt1 and Xfz3, which are co-expressed in the dorsal neural tube. Xfz3 is specifically required for Xwnt1-mediated neural crest induction in explants. Furthermore, Xfz3 is required for the formation of neural crest in the developing vertebrate embryo. Finally, this work suggests a functional interaction between endogenous Wnt and frizzled family members in the developing vertebrate embryo.

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