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Frizzled7 mediates canonical Wnt signaling in neural crest induction

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Abstract

The neural crest is a multipotent cell population that migrates from the dorsal edge of the neural tube to various parts of the embryo where it differentiates into a remarkable variety of different cell types. Initial induction of neural crest is mediated by a combination of BMP, Wnt, FGF, Retinoic acid and Notch/Delta signaling. The two-signal model for neural crest induction suggests that BMP signaling induces the competence to become neural crest. The second signal involves Wnt acting through the canonical pathway and leads to expression of neural crest markers such as slug. Wnt signals from the neural plate, non-neural ectoderm and paraxial mesoderm have all been suggested to play a role in neural crest induction. We show that *Xenopus* frizzled7 (Xfz7) is expressed in the dorsal ectoderm including early neural crest progenitors and is a key mediator of the Wnt inductive signal. We demonstrate that Xfz7 expression is induced in response to a BMP antagonist, noggin, and that Xfz7 can induce neural crest specific genes in noggin-treated ectodermal explants (animal caps). Morpholino-mediated or dominant negative inhibition of Xfz7 inhibits Wnt induced Xslug expression in the animal cap assay and in the whole embryo leading to a loss of neural crest derived pigment cells. Full-length Xfz7 rescues the morpholino-induced phenotype, as does activated β-catenin, suggesting that Xfz7 is signaling through the canonical pathway. We therefore demonstrate that Xfz7 is regulated by BMP antagonism and is required for neural crest induction by Wnt in the developing vertebrate embryo.

Keywords: Neural crest; *Xenopus*; Frizzled; Wnt; Xfz7; Slug; BMP; Noggin

Introduction

The neural crest is a multipotent population of cells that arise at the border between the neural ectoderm and the non-neural ectoderm. As the neural plate folds during neurulation, the neural crest cells undergo an epithelial to mesenchymal transition. They delaminate from the dorsal neural tube in an anterior–posterior wave and migrate to various parts of the embryo, where they differentiate into a large variety of cell types in the body including most of the craniofacial skeleton, neurons and glial cells of the peripheral nervous system, pigment cells and neuroendocrine cells of the adrenal medulla (Basch et al., 2004; Huang and Saint-Jeannet, 2004).

Induction of the neural crest starts during gastrulation, continues until neural tube closure and is thought to be mediated by a complex series of interactions between the neural plate, non-neural ectoderm and paraxial mesoderm. Support for this model has come particularly from experiments in *Xenopus* and chick (Huang and Saint-Jeannet, 2004). For example, in *Xenopus*, recombining neural plate with non-neural ectoderm led to subsequent expression of Xslug, a neural crest specific transcription factor, at the ectoderm/neural boundary (Mancilla and Mayor, 1996). Removing the presumptive paraxial mesoderm of *Xenopus* embryos leads to a decrease in Xslug expression (Bonstein et al., 1998; Marchant et al., 1998) and in chick, explanting neural plate into non-neural ectoderm led to induction of neural crest (Dickinson et al., 1995).

A number of transcription factors have been shown to be required in the formation of the neural crest. These include Twist, Snail, Zic3, Pax3, Pax7, Msx1, Sox10, FoxD3, Sox9, cMyc, Id3, Dlx and AP2a (Basch et al., 2004, 2006; Huang and Saint-Jeannet, 2004; Steventon et al., 2005). One of the earliest neural crest specific genes to be expressed and shown to be required for neural crest development is the transcription factor Xslug (Mayor et al., 1995). Xslug is therefore a reliable indicator of neural crest induction.

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The signals controlling expression of these factors and thus neural crest development are not fully understood. Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Wnt, Retinoic acid (RA) and Notch/Delta signaling have all been implicated with multiple signals leading to the initial induction, expansion, maintenance and differentiation of neural crest cells (Aybar and Mayor, 2002; Baker and Bronner-Fraser, 1997; Villanueva et al., 2002).

Wnt signaling is critically important in neural crest induction and migration (De Calisto et al., 2005). We have investigated the role of Wnt signaling and in particular the Wnt receptor *Xenopus* frizzled7 (Xfz7) in neural crest induction. Wnts are secreted glycoproteins that control development in organisms ranging from hydra to man. Wnts signal through the seven pass transmembrane domain receptors called the frizzleds. The response to Wnts is mediated by different pathways including the canonical, β-catenin-dependent pathway, the planar cell polarity (PCP) pathway and the PKC/Ca^{2+} pathway (reviewed by Bejsovec, 2005; Wodarz and Nusse, 1998). We have previously cloned Xfz7 and shown its expression in the dorsal ectoderm, during gastrulation in the presumptive neural ectoderm, during neurulation in the neural plate, neural crest, mesoderm and heart field and later in the brain, neural tube, migrating neural crest, otic vesicle, eye and pronephric duct (Wheeler and Hoppler, 1999). Xfz7 has been shown to be involved in the regulation of different cellular processes during early stages of embryogenesis including convergent extension movements, tissue separation during gastrulation, anterior–posterior patterning, axis formation and mesoderm induction (Djiane et al., 2000; Medina et al., 2000; Sumanas and Ekker, 2001; Sumanas et al., 2000; Wheeler et al., 2000; Winklbauer et al., 2001). However, the potential role of Xfz7 in neural crest formation suggested by the expression pattern has not yet been investigated.

A number of different Wnts have been implicated in neural crest induction both from expression patterns and from functional work (Yanfeng et al., 2003). Overexpression of Wnts 1 and 3a leads to an expansion of neural crest in *Xenopus* embryos (Saint-Jeannet et al., 1997). An ectodermal explant obtained by cutting the animal cap from a blastula stage embryo injected with a neuralizing signal such as noggin encoding RNA will only express neural markers such as Sox2. However, if it is co-injected with Wnt RNA such as Wnt1, Wnt 3a, Wnt7b or Wnt8, it will also lead to expression of a range of neural crest markers such as Slug, Pax3 and Zic2 (Bang et al., 1999; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997). Of these Wnts, Xwnt1 and Xwnt3a are expressed in the lateral neural plate and neural folds and later on in the dorsal neural tube, Xwnt7b is expressed in the neural and non-neural ectoderm in the early *Xenopus* neurula and becomes restricted to the dorsal neural tube after neural tube closure, and Xwnt8 is expressed in the paraxial mesoderm. Inhibiting Wnt signaling can block neural crest induction (LaBonne and Bronner-Fraser, 1998; Bastidas et al., 2004; Deardorff et al., 2001). In chick embryos, COS cells expressing a dominant negative form of Wnt1 placed adjacent to the neural folds prevent the expression of Slug (Garcia-Castro et al., 2002). Also in chick, Wnt6 has been shown to be expressed in the ectoderm next to the boundary with the neural plate and has thus been suggested as a neural crest inducer, though no functional evidence has yet been reported (Garcia-Castro et al., 2002; Schubert et al., 2002). The canonical pathway is thought to mediate these inductive signals as overexpression of downstream components of this pathway, such as β-catenin or LRP6, induces neural crest markers (LaBonne and Bronner-Fraser, 1999; Tamai et al., 2000). The role of the canonical pathway is confirmed by loss of function analyses with dominant negative forms of LRP6 (Tamai et al., 2000), Xwnt8 (LaBonne and Bronner-Fraser, 1998), Tcf3 (Lewis et al., 2004) and *Xenopus* frizzled3 (Xfz3, Deardorff et al., 2001), all of which block Xslug expression. Moreover, the Tcf/LEF family of transcription factors, which are the effectors of the canonical pathway, appear to bind to and directly regulate the Xslug promoter (Sakai et al., 2005; Vallin et al., 2001).

Initial neural crest induction in *Xenopus* has been suggested to occur in a two-step process. First, a gradient of BMP signaling specifies epidermal, neural and border between the ectoderm and neural ectoderm (prospective neural crest) in the ectoderm. The ectoderm at the border is competent to respond to a signal provided by Wnt and/or FGF which initiates and maintains neural crest induction (Bastidas et al., 2004; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Mayor et al., 1997). Wnt signaling induces the expression of Pax3 at the neural plate border (Monsoro-Burq et al., 2005). Pax3 in association with Msx1 and further Wnt signaling initiates Xslug expression and neural crest differentiation (Monsoro-Burq et al., 2005; Sato et al., 2005). Wnt signaling is thus important in the initial expression of Pax3 and the subsequent induction of the neural crest at the neural plate border.

Recently, Wnt signaling via the PCP pathway rather than the canonical pathway has also been shown to be important for neural crest migration (De Calisto et al., 2005). It was shown that a mutant Dsh which inhibits the canonical pathway can prevent neural crest induction but a mutant form of Dsh that inhibits the PCP pathway specifically led to defects in the migration of the neural crest without affecting the initial induction. Wnt11, which is known to signal through non-canonical and canonical pathways (Heisenberg et al., 2000; Tao et al., 2005), was shown to be necessary for neural crest migration. Furthermore, Xfz7 is expressed in the cells, which respond to this signal (De Calisto et al., 2005).

Cells competent to become neural crest would be expected to express the frizzled receptors for one or more of the Wnts involved in induction and migration. It has been shown that ectopic expression of Xfz3 can induce Xslug and that a dominant negative form of Xfz3 and morpholino knockdown can inhibit Xslug expression (Deardorff et al., 2001). Here, we present evidence that Xfz7 is expressed in cells competent to become neural crest unlike Xfz3 and that Xfz7 expression can be regulated by noggin. We show that Xfz7 can induce Xslug in a dose-dependent manner in explant assays and that a dominant negative form and morpholino can specifically inhibit neural crest marker expression in explant assays and in intact embryos without affecting the neural marker Xsox2. We show that Xfz7

is acting via the canonical pathway and can mediate signals from Wnts, suggested to function in neural crest induction. Our data therefore uncover a crucial function of Xfz7 in neural crest induction, via the canonical Wnt signaling pathway.

Materials and methods

Embryos, in vitro mRNA synthesis, morpholino and microinjection

Xenopus laevis embryos were obtained as previously described (Harrison et al., 2004) and were staged according to normal table of Nieuwkoop and Faber (1994). Capped mRNAs were synthesized according to the manufacturer's instructions of SP6 mMESSAGE mMACHINE Ambion kit. Morpholino antisense oligonucleotides (MOs) were synthesized by Gene Tools using sequences reported elsewhere: Xfz7 MO1 (5′-CCAACAAGTGATCTCTGGA-CAGCAG-3′) (Winklbauer et al., 2001), Xfz7 MO2 (5′-GCGGAGTGAGCA-GAAATCGGCTGA-3′) and Xfz7 MO3 (5′-CCGGCTCCAACAAGTGATCT-CTGG-3′) (Sumanas and Ekker, 2001). All morpholinos were subjected to *in vitro* translation assay before use (TNT coupled reticulocyte lysate system, Promega). mRNAs or MOs were co-injected with 250 pg *Lac-Z* (kind gift from Dr. Maggie Walmesly) as a lineage tracer into 2-, 4-, 16- or 32-cell stage using 10 nl calibrated needles. β-galactosidase activity was detected using Red-Gal (Research Organics) as a substrate. pCS2Xfz7FL and pCS2Xfz7CRD were made by subcloning a *Cla*1–*Xho*1 fragment out of pHSXfz(S/B)HSG2 (Wheeler et al., 2000) and pHSXfz7(CRD)HSG into pCS2+ cut with *Cla*1–*Xho*1. Other constructs used in this study were kind gifts of Peter Klein (Xfz3 full-length and Xfz3 CRD), Oliver Destree (Xwnt1), Richard Harland (Xwnt7b), Jan Christian (Xwnt8), Yoshiki Sasai (Chordin), Dave Hsu (Noggin) and Randall Moon (β-catenin).

To avoid titration of the Xfz7 morpholino by RNA in the rescue experiment, site-directed mutagenesis of the full coding sequence of Xfz7 was carried out by inserting a *Kpn*1 site at the 5′ morpholino target sequence of pCS2Xfz7FL. We called this Xfz7SDM.

In situ hybridization and histology

In situ probes used in this study were kind gifts of Michael Sargent (Xslug and Twist), Jean-Pierre Saint-Jeannet (Snail and Sox10), Yoshiki Sasai (Sox2 and FoxD3) and Peter Klein (Xfz3). RNA probes were labeled with either digoxigenin or fluorescein (Roche) as previously described (Melton et al., 1984). Single (Harland, 1991) or double (Knecht et al., 1995) *in situ* hybridization was also carried out as previously described (Harrison et al., 2004). In double *in situ* hybridization, DIG-labeled probes were visualized with NBT/BCIP or BCIP and fluorescein-labeled probes were visualized with fast red (Roche). For frozen sectioning, embryos were processed in 30% sucrose, embedded in OCT and sectioned at 15–20 μm.

Animal cap assay, RNA isolation and RT-PCR

The neural crest induction assay in the animal cap explants was carried out as previously described (Saint-Jeannet et al., 1997). Briefly, embryos were injected at one-cell stage in the animal pole with 500 pg mRNA for noggin alone or in combination with Xwnts, Xfrizzleds and/or Xfz7 MO2 at concentrations indicated in the figure legends. At stage 6, embryos were devitellinized and at stage 9 about 15–20 animal caps for each injection were cut then incubated at 18°C until their siblings reached stage 19. Total RNA extraction was carried out according to the manufacturer's instructions using the RNeasy Mini kit (QIAGEN). RT-PCR was performed as previously described (Mizuseki et al., 1998). The primers' sequence is as previously reported: Histone H4 (Perry et al., 1985), Xslug (http://template.bio.warwick.ac.uk/staff/hwoodland/HRW3), Pax3 and Zic2 (Sato et al., 2005), Xsox2 (Mizuseki et al., 1998), Xbra (XMMR homepage), Xfz7 and En-2 (Brown et al., 2000) and Xfz3 (Deardorff et al., 2001). For each RT-PCR analysis, Histone H4 was used as a control and the injected animal caps were checked for the neural and mesodermal induction using Sox2 and Xbra markers respectively. Non-injected animal caps were used in each experiment as a negative control.

Results

Xfz7 is expressed in early neural crest

We have previously shown that Xfz7 is expressed in the migrating cranial neural crest (Wheeler and Hoppler, 1999, see also De Calisto et al., 2005). We therefore wanted to determine when Xfz7 was first expressed during neural crest development (Fig. 1). RT-PCR showed Xfz7 to be maternally expressed in the oocyte and at stages 1 and 4. Expression increased after the mid-blastula transition and was maintained throughout gastrulation and neurulation (Fig. 1A). RT-PCR for the early neural crest marker Pax3 showed strong expression from stage 12, while Xslug (Mayor et al., 1995) showed expression from stage 13 onwards (Fig. 1A). Xfz7 is thus expressed at the time when neural crest begins to be induced. Interestingly, Xfz3 expression at stage 12 was very weak and only came on strong at stage 13 (Fig. 1A). For a frizzled receptor to respond to a Wnt signal and initiate neural crest induction, it would need to be expressed in the relevant cells. To determine whether Xfz7 was expressed in the right spatial pattern to be involved in neural crest induction, we used wholemount double in situs to see whether Xfz7 expression overlapped with that of Xslug. Fig. 1B shows that Xfz7 expression in the neural plate and dorsal mesoderm (in red) overlapped with the initial expression of Xslug (in blue) at stage 13. By stage 18, expression was seen in epidermis, lateral mesoderm and in neural plate including the placodes except for the most anterior forebrain area. Xfz7 continued to be expressed in Xslug positive cells just before migration starts (Fig. 1Ci and Cii). Xfz3, which has been implicated in neural crest induction (Deardorff et al., 2001), did not overlap with Xslug expression at stage 13 (Fig. 1D, F and G) or 18 (Fig. 1Ei and ii) and was mostly restricted to the developing CNS. Thus, Xfz7 shows a wider expression than slug but is expressed at the right time and place to mediate Wnt induction of neural crest.

Xfz7 can induce neural crest markers

In order to determine if Xfz7 is necessary for neural crest induction, we used a dominant negative form of Xfz7 (Xfz7CRD) and an Xfz7 morpholino (Xfz7MO) to interfere with its function. The dominant negative form of Xfz7 was generated by deleting the transmembrane domains and cytoplasmic domain (see Materials and methods for details). The resulting protein contained the signal sequence and cysteine rich domain (CRD). In order to determine if this construct was functional, we injected it into the dorsal marginal zone (DMZ) of 4-cell stage embryos. The embryos developed typical convergent extension phenotypes seen previously with Xfz7 dominant negative constructs (Fig. 2A and Sumanas et al., 2000). This phenotype could be rescued using full-length Xfz7 (Fig. 2B). At higher concentrations, Xfz7 was also capable of inducing convergent extension phenotypes (Fig. 2C and D). The Xfz7MO used in this study has been described before and corresponds to MO2 in Sumanas and Ekker (2001). Fig. 2E

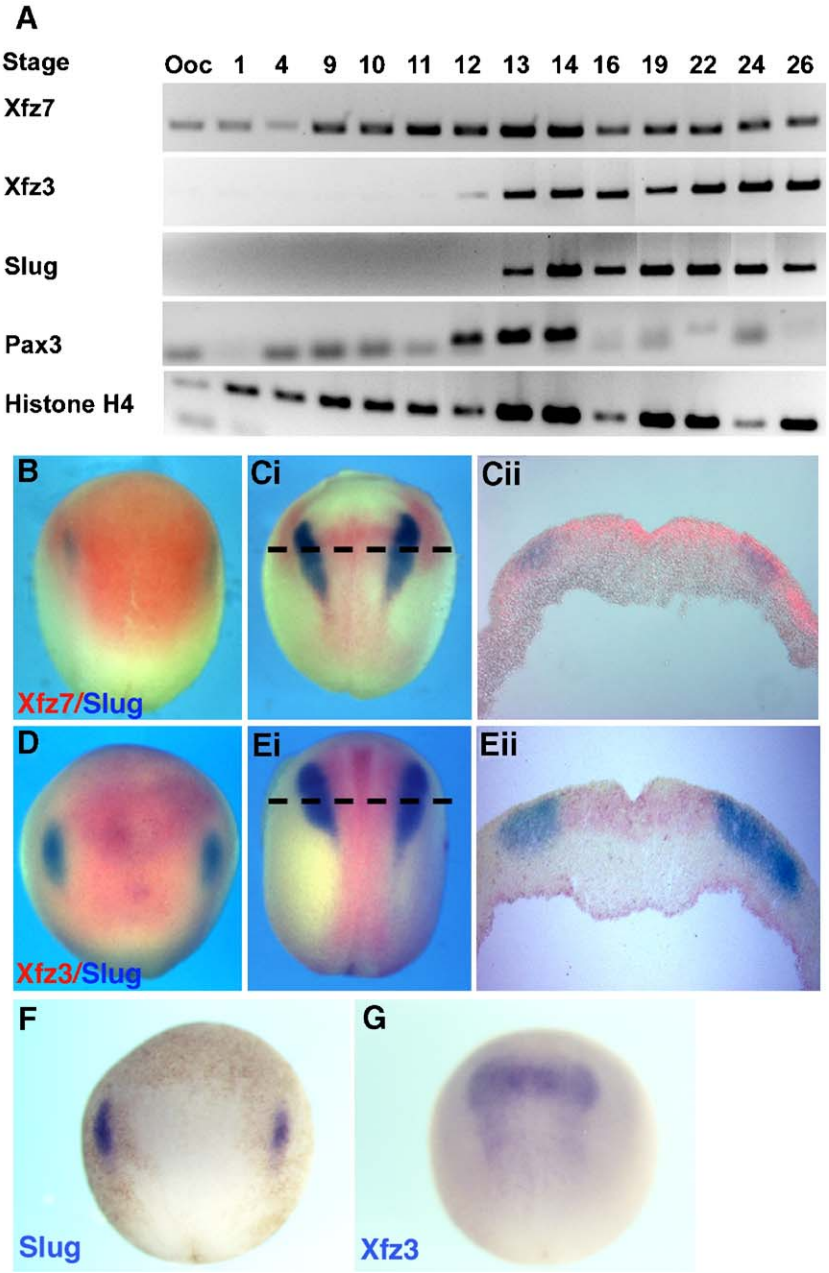

Fig. 1. Xfz7 rather than Xfz3 is expressed in the early neural crest. (A) Embryos were harvested at the stages indicated and expression of Xfz7, Xfz3, Slug and Pax3 was assessed by RT-PCR. Histone H4 was used as a loading control. (B–E) Double label in situ expression of Xfz7 and Xfz3 with Xslug at early neurula (stage 13, B and D) and late neurula stage (stage 18, Ci and Ei). Xfz7 and Xfz3 (red) and Slug (purple). Cii and Eii are frozen sections cut at the level shown in panels Ci and Ei. (F and G) Wholemount in situ expression of panels F slug and G Xfz3 on stage 13 embryos.

shows the sequence of MO1, 2 and 3. We have called the two pseudoalleles of Xfz7, Xfz7A and B, respectively. *In vitro* translation showed that MO2 was able to reduce substantially Xfz7A translation (Fig. 2F, lane 3 compared with lane 1). Neither MO1 (Winklbauer et al., 2001) nor MO3 (MO1 in Sumanas and Ekker, 2001) described previously had an effect on Xfz7 in this assay (Fig. 2F, lanes 2 and 4). The standard Genetools control MO had no effect on Xfz7 translation (Fig. 2F, lane 5). Fig. 2G shows that increasing concentrations of MO2 inhibited Xfz7A translation. Based on these results, we decided to use MO2 to inhibit convergent extension movements to demonstrate efficacy (for an example, see Fig. 6C and D). MO1 also gave convergent extension phenotypes while MO3 did not affect convergent extension movements (data not shown).

It has previously been shown that a combination of Wnt and noggin RNA can induce expression of Slug when injected into animal cap explants. We therefore tested if Xfz7 could induce neural crest markers in similar animal cap assays. We found that Xfz7 can induce Xslug and other neural crest markers (Pax3 and Zic2) in animal caps neuralized with noggin in a way similar to Wnt1 and Xfz3 (Fig. 3A, lanes 2–4). These results show Xfz7 to be a potent inducer of neural crest in animal caps. None of these animal caps showed any mesodermal induction as shown

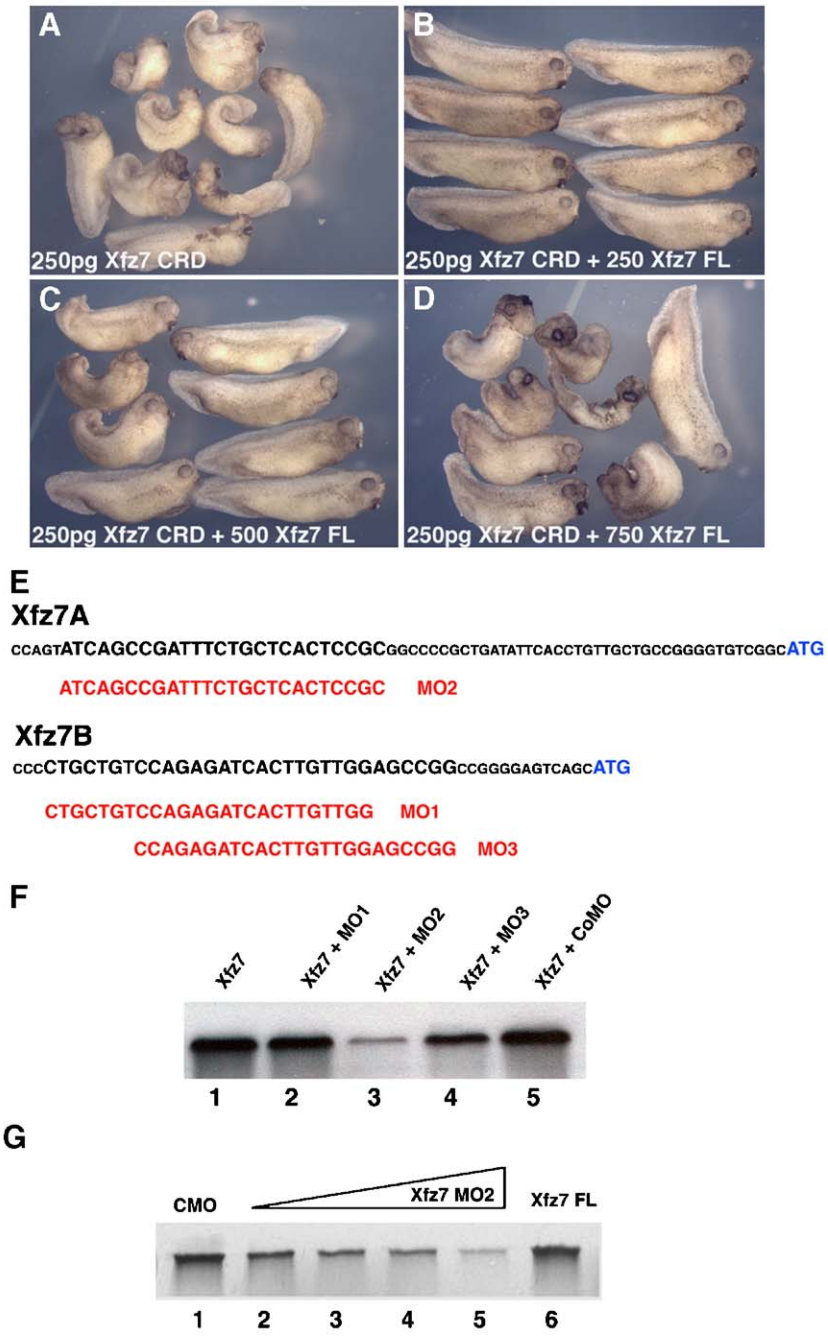

Fig. 2. Xfz7 is important for convergent extension movements. (A) injection of 250 pg of Xfz7 CRD leads to boat shape embryos typical of defects in convergent extension movements. (B) Co-injection of 250 pg Xfz7 CRD and 250 pg Xfz7. Full-length leads to normal looking embryos. (C and D) Injection of increasing concentrations of Xfz7 leads to increasing convergent extension defects. (E) Diagram showing the binding sites for MO1, 2 and 3 to Xfz7A (Wheeler and Hoppler, 1999) and Xfz7B (Medina et al., 2000; Sumanas et al., 2000). (F and G) mRNA and protein were prepared from Xfz7 plasmid using an *in vitro* transcription translation system. ^{35}S-labeled translation products were analyzed on SDSPAGE gels. (F) Translation of Xfz7 RNA (lane 1) was decreased in the presence of MO2 (lane 3) but not by MO1 (lane 2), MO3 (lane 4) and control MO (lane 5). The results shown are representative for at least 3 independent experiments. (G) Translation of Xfz7 RNA was decreased as the amount of MO2 was increased. Lane 1, control MO, lanes 2–5, 20, 40, 60 and 80 ng MO2, lane 6, Xfz7 on its own.

by absence of Xbra expression. Interestingly, Xfz7 could also turn on the expression of the posterior neural plate marker Engrailed-2 (Fig. 3A, lane 4) similar to Xwnt3A (Villanueva et al., 2002). If Xwnt1 can induce neural crest markers such as slug, it presumably must be binding to a frizzled receptor expressed in the animal cap. We therefore tested whether Xfz7 or Xfz3 was present in the neuralized animal caps. Fig. 3B shows that both Xfz7 and Xfz3 were present at low levels in uninjected animal caps but are upregulated in animal caps injected with noggin. Interestingly, none of the Wnts we tested including Xwnt1, Xwnt8 (Fig. 3B) and Xwnt6 (data not shown) was upregulated in the neuralized caps. Because Xfz7 is widely expressed including in areas that do not generate neural crest, we asked whether Slug induction was sensitive to variations in

Xfz7 concentration. We found that highest slug expression occurred at an intermediate concentration of Xfz7 (Fig. 3C). Too little (250 pg) or too much (1 ng) Xfz7 resulted in more limited slug expression.

Xfz7 is required for neural crest induction in vivo

Our data demonstrated that Xfz7 can induce neural crest in neuralized animal caps. Furthermore, Xfz7 was expressed at the right time and place to mediate neural crest induction in the whole embryo. We therefore determined whether it could play a direct role in this process *in vivo*. We injected Xfz7 CRD RNA or Xfz7MO into one blastomere of 2-, 4-, 8- or 16-cell stage embryos and let them develop until early and late neurula stages. We then carried out in situ hybridization to detect Slug expression (Fig. 4). In all cases, we co-injected LacZ RNA, which was then visualized with Red Gal to detect the injected side. Figs. 4B and C show that Xfz7 CRD and MO caused a downregulation of Slug expression. Xfz3 CRD, like Xfz7, could downregulate slug (Fig. 4E). We obtained similar results when 8- or 16-cell stage embryos were injected with Xfz7CRD or MO in the dorsal–animal hemisphere cells that are fated to become neural crest (Figs. 4H, I and K; Moody, 1987). In these experiments, overexpression of Xfz7 did not induce ectopic Xslug while Xfz3 did (compare Figs. 4A and D). Injections of Xfz10 MO or the standard control MO had no effect on Xslug expression (Figs. 4F, J, Fig. 5 left hand panels). Injection of Xfz7MO into the DMZ of an 8-cell stage embryo led to an open blastopore and open neural tube as expected. In some cases, bending of the neural tube was observed as a manifestation of the convergent extension phenotypes caused by disruption of Xfz7 (data not shown). Xslug expression, however, remained normal, suggesting the morphogenetic defects caused by Xfz7 did not affect neural crest induction (Fig. 4G). Injections into the D1.2 blastomere of 16-cell embryos did not show convergent extension phenotypes at stage 18 (Fig. 4I). Expression of Xslug was downregulated or lost at stage 13 after injection with Xfz7CRD or Xfz7MO (Figs. 4H and K). This suggests that we may be affecting initiation of Xslug expression versus maintenance. The injected embryos looked morphologically normal at stage 13. Expression analysis of the neural plate marker Xsox2 in these embryos showed that the neural plate looked normal despite the lack of Xfz7 and loss of Xslug expression, indicating that the effect was specific for neural crest (Fig. 4L). In fact, later on in neural development, Xsox2 expression expanded in areas with decreased neural crest (Figs. 5G–I). Similar results were obtained for other neural crest markers such as FoxD3, Sox10, Sox9, Snail and Twist (Figs. 5A–F and J–R) where injection of Xfz7MO or Xfz7CRD led to a downregulation of their expression. Thus, interfering with the function of Xfz7 clearly inhibited or prevented the initial expression of slug, suggesting that this receptor is important for neural crest induction.

Inhibition of Xfz7 did not just lead to a decrease in early expression of neural crest markers. Embryos injected with the Xfz7MO into both blastomeres of the animal pole of a 2-cell stage embryo when allowed to develop to stage 40 showed a greatly reduced number of neural crest derived pigment cells in their flanks (Figs. 6E and F) compared to control embryos (Figs.

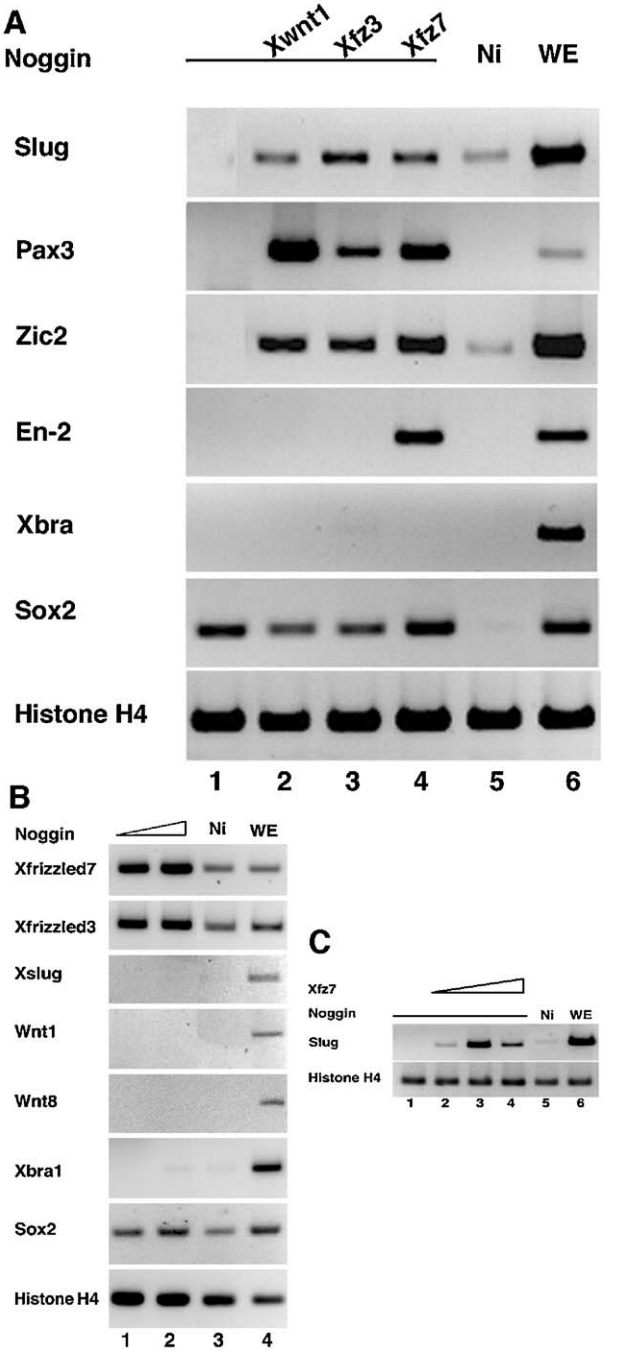

Fig. 3. Xfz7 induces neural crest markers in animal caps. (A) Xfz7 induces the same neural crest markers as Xwnt1 and Xfz3 shown by RT-PCR analysis of animal caps expressing noggin (500 pg) alone (lane 1) or with Xwnt1 (100 pg, lane 2), Xfz3 (1 ng, lane 3) or Xfz7 (500 pg, lane 4). (B) 500 pg and 750 pg of noggin injected into animal caps induces expression of Sox2, Xfz7 and Xfz3 but not Xslug, Wnt1, Wnt8 and Xbra1 (lanes 1 and 2) compared with the non-injected control (Ni, lane 3). WE in lane 4 stands for whole embryo. (C) RT-PCR analysis of slug expression is shown for animal caps injected with noggin (500 pg) alone (lane 1) or with increasing amounts of Xfz7 250 pg (lane 2), 500 pg (lane 3), 1 ng (lane 4). 'Ni' indicates animal cap from uninjected embryo and 'WE' indicates RNA made from whole embryos at stage 19.

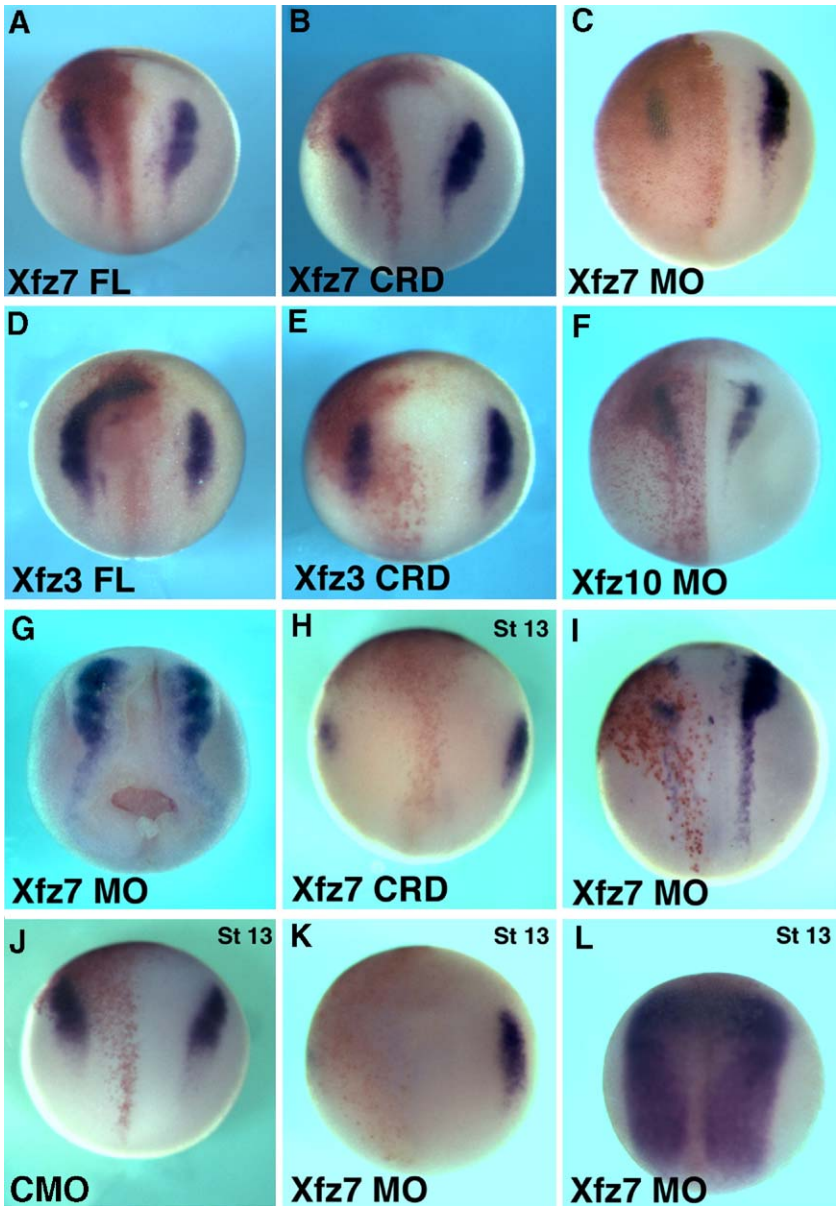

Fig. 4. Analysis of Xfz7 function in neural crest formation in whole embryos. Embryos were injected with RNA or MO at the 2-cell (A–F), 8-cell (G, H, J–L) or 16-cell stage (I) into the animal pole region of one blastomere except for (G) where the embryo was injected twice into the DMZ. RNA's or MO's used were: (A) Xfz7 full-length (1 ng), (B) Xfz7CRD (1 ng), (C) Xfz7MO (35 ng), (D) Xfz3 full-length (1 ng), (E) Xfz3CRD (1 ng), (F) Xfz10MO (50 ng), (G) Xfz7MO (60 ng), (H) Xfz7CRD (500 pg). (I) Xfz7MO (40 ng) into the D1.2 blastomere, (J) CMO (35 ng), (K) Xfz7MO (35 ng), (L) Xfz7MO (35 ng). All injections contained the lineage tracer β-galactosidase (250 pg). Thus, the red nuclear β-galactosidase staining indicates the injected side. The dark purple indicates Xslug expression except (L) where it is sox2 expression. All embryos are stage 18 except for panels H, J–L which are stage 13. All embryos are dorsal views with anterior to the top.

6A and B) and to embryos injected with Xfz7MO in the DMZ (Figs. 6C and D).

To test the specificity of the Xfz7 MO, we set out to rescue the phenotype with full-length Xfz7 which was modified to prevent the MO from binding Xfz7SDM (see Materials and methods). This did not affect the amino acid sequence of the Xfz7 protein and was shown by *in vitro* translation to be insensitive to the Xfz7MO (data not shown). This modified Xfz7 retained its function and resulted in convergent extension phenotypes when injected into the DMZ of 4-cell stage embryos (data not shown). When co-injected with the Xfz7 MO into the animal pole of 2-cell stage embryos, the Xfz7SDM RNA was able to rescue the downregulation of Xslug with 86% of embryos showing normal slug expression compared with 22% showing normal expression when just the Xfz7MO was injected (Fig. 7A).

Xfz7 mediates neural crest induction via the canonical Wnt pathway

Xfz7 has been suggested to activate both the canonical and non-canonical Wnt signaling pathways (Medina et al., 2000). It has also been previously shown that canonical Wnt signaling can

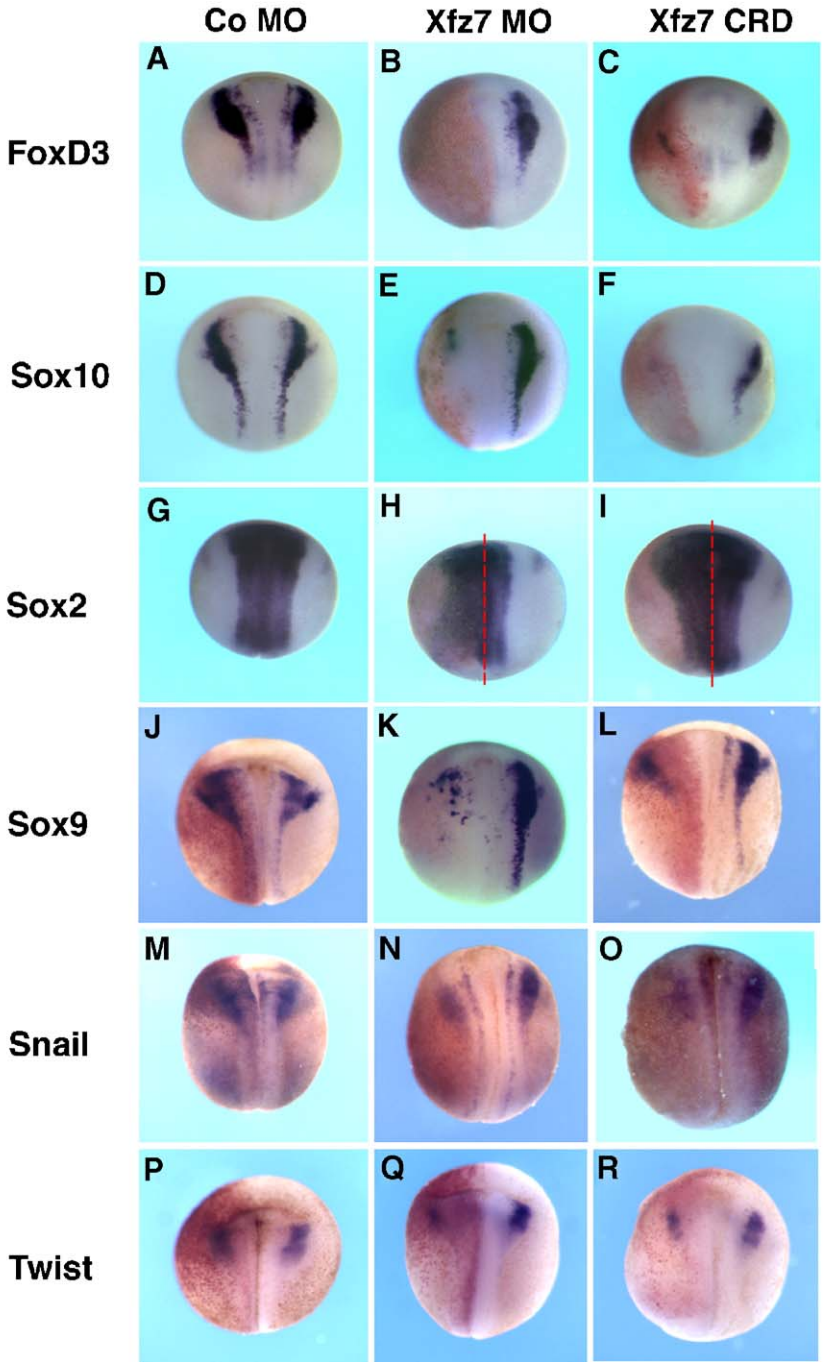

Fig. 5. Loss of Xfz7 function leads to downregulation of neural crest gene expression in whole embryos and expansion of neural gene expression. Embryos were injected at the 2-cell stage (except K, 8 cell stage) with RNA in the animal pole of one blastomere. MOs or RNA' used were standard control MO (60 ng) (A, D, G, J, M, P), Xfz7MO (60 ng except (K) 40 ng) (B, E, H, N, Q) and Xfz7CRD (1 ng) (C, F, I, L, O, R). All injections contained the lineage tracer β-galactosidase (250 pg). Thus, the red nuclear β-galactosidase staining indicates the injected side. The dark purple indicates the expression of FoxD3 (A–C), Sox10 (D–F), Sox2 (G–I), Sox9 (J–L), Snail (M–O) and Twist (P–R). All embryos are dorsal views with anterior to the top. The dashed line in panels H and I indicates the embryonic midline.

induce neural crest (Deardorff et al., 2001; Wu et al., 2005). To determine which pathways are involved, we attempted to rescue the effect of Xfz7MO with constitutively active β-catenin. Injection of 35 ng of Xfz7MO into one blastomere of a 2-cell embryo leads to a downregulation of Xslug expression. Co-injection with β-catenin led to almost normal levels of Xslug expression (Fig. 7B). This suggests that in the context of neural crest induction Xfz7 could be signaling via the canonical pathway.

Xfz7 morpholino can block Wnt-mediated neural crest induction

Neuralized animal caps express elevated levels of both Xfz7 and Xfz3 (Fig. 3B) but do not activate neural crest markers possibly because ligand is in limited supply. We tested which Wnt or Wnts might be interacting with Xfz7 to control neural crest induction using the animal cap assay. Wnts known or thought to induce neural crest are Wnt1,

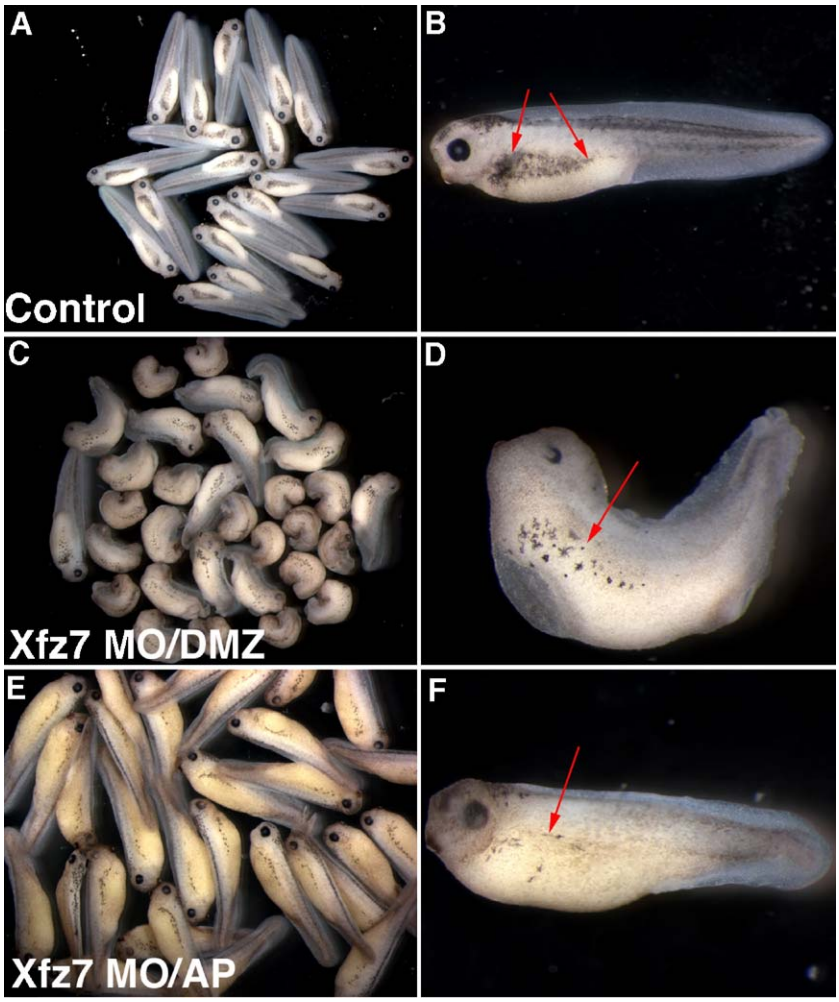

Fig. 6. Downregulation of Xfz7 expression leads to the loss of melanocytes in whole embryos. (A and B) Control embryos. The arrows show the neural crest derived melanocytes concentrated in the trunk. (C and D) Injection of Xfz7 MO (60 ng) into the DMZ of a 4-cell stage embryo results in decreased melanocytes (arrow) and morphogenetic defects. (E–F) Injection of Xfz7 MO (60 ng) into the animal pole region of both blastomeres of a 2-cell stage embryo results in absent melanocytes (arrow).

Wnt7b and Wnt8. Co-injection of these Wnts into neuralized animal caps resulted in Xslug expression (Fig. 8, lanes 1, 3 and 5) and neural crest induction. We then co-injected Xfz7MO and found that Xslug expression was reduced (Fig. 8, lanes 2, 4 and 6), illustrating that the Xfz7 MO could indeed inhibit Xwnt1-, Xwnt7b- and Xwnt8-mediated neural crest induction.

Discussion

In vertebrates, neural crest induction is controlled by a number of signaling pathways including BMP, FGF, Notch, RA and Wnt (Huang and Saint-Jeannet, 2004). Wnt signaling has been shown to play multiple roles in neural crest development in various organisms and different Wnt pathways seem to mediate different effects. In this paper, we show that Xfz7 is a very good candidate to mediate some of these Wnt signals in the *Xenopus* embryo. Firstly, Xfz7 is expressed very early during neural crest development. Secondly, the dominant negative form of Xfz7 or Xfz7MO inhibits expression of early neural crest markers but not neural markers. This inhibition can be rescued by full-length Xfz7, demonstrating that the effect is specific and suggesting that Xfz7 is crucial for early neural crest specification. Thirdly, Xfz7MO inhibition of the neural crest can be rescued by its downstream target β-catenin, suggesting that Xfz7 signals through the canonical pathway.

Specification of the neural plate boundary and induction of neural crest is controlled by the canonical Wnt pathway (Wu et al., 2005). Migration of the neural crest is controlled by the non-canonical or PCP pathway (De Calisto et al., 2005). For the induction of neural crest, a number of different Wnts have been suggested to play a role. These include Wnt1, Wnt6, Wnt7b and Wnt8c (Yanfeng et al., 2003). We show that Xfz7 might interact with the neural crest inducers Xwnt1, Xwnt7b and Xwnt8. To date, the only Wnt receptor suggested to mediate these signals for neural crest induction has been Xfz3. However, it has not been shown conclusively whether or not Xfz3 is expressed in Slug expressing cells.

We have previously reported Xfz7 to be expressed in the developing neuroectoderm and later in migrating neural crest

(Wheeler and Hoppler, 1999). We have now shown using wholemount double in situ hybridization that Xfz7 is expressed in the early neural crest progenitors and colocalizes with Xslug. Xfz3 is expressed in the neural plate (Shi et al., 1998; Deardorff et al., 2001 and Figs. 1D–G) but does not seem to overlap with the areas expressing slug though it is possible low levels of Xfz3 could be present. Xfz3 is expressed early in neural crest development alongside slug expression but is not expressed at high levels when Pax3 which is an earlier marker for neural crest induction is being expressed (Fig. 1A), thus suggesting that it may not be involved in early induction events. In chick, frizzled7 is expressed in the neural folds adjacent to Wnt6 (Dr. S. Dietrich, personal communication), which has been postulated as the ectodermal neural crest inducer (Garcia-Castro et al., 2002; Schubert et al., 2002). Preliminary work with *Xenopus* Wnt6 has shown that it is not expressed until after neural crest induction has occurred (D. Heine and Dr. S. Hoppler, personal communication).

Inhibition of Xfz7 activity either by injection of a dominant negative form or by a morpholino led to the loss of a number of early neural crest markers (Xslug, FoxD3, Sox10, Sox9, Snail and Twist) while at the same time expanding the expression of the neural marker Xsox2 (Figs. 5G–I). Additionally, Xfz7MO prevented the production of melanocytes (pigment cells), which are derived from neural crest (Fig. 6). The inhibition of Xslug induction by Xfz7MO was rescued by a modified full-length Xfz7 (Fig. 7A). The Xfz7-induced loss of neural crest phenotype could also be rescued by β-catenin (Fig. 7B). Xfz7 has been suggested to signal through both the canonical and non-canonical pathways (Medina et al., 2000; Sumanas and Ekker, 2001). Our results suggest that in this context Xfz7 is working through the canonical β-catenin-dependent pathway.

In the animal cap assay, full-length Xfz7 can induce the neural crest markers Xslug, Xtwist, Xpax3 and Xzic2. Surprisingly, injection of full-length Xfz7 into the whole embryo did not lead to increased or ectopic expression of neural crest markers such as Xslug. Xwnt1, Xfz3 and β-catenin when ectopically expressed all lead to increased slug expression (Figs. 4D, 7B and Deardorff et al., 2001). We have clearly shown our full-length Xfz7 to be functional through its ability to inhibit convergent extension movements (Figs. 2C and D and data not shown). There are a number of reasons why overexpression of Xfz7 could lead to activation of neural crest markers in the animal cap. Forced dimerization of the receptor is one possibility, and it has been shown that Xfz3 dimerization leads to signaling via the Wnt/β-catenin pathway (Carron et al., 2003). It has also been found that Xfz7 is able to dimerize (Kaykas et al., 2004). Another possible explanation is that noggin could induce expression of a Wnt ligand capable of signaling through Xfz7. We have shown that Wnt1 and Wnt8 are not upregulated in noggin-treated animal caps (Fig. 3B) though other Wnts still need to be tested.

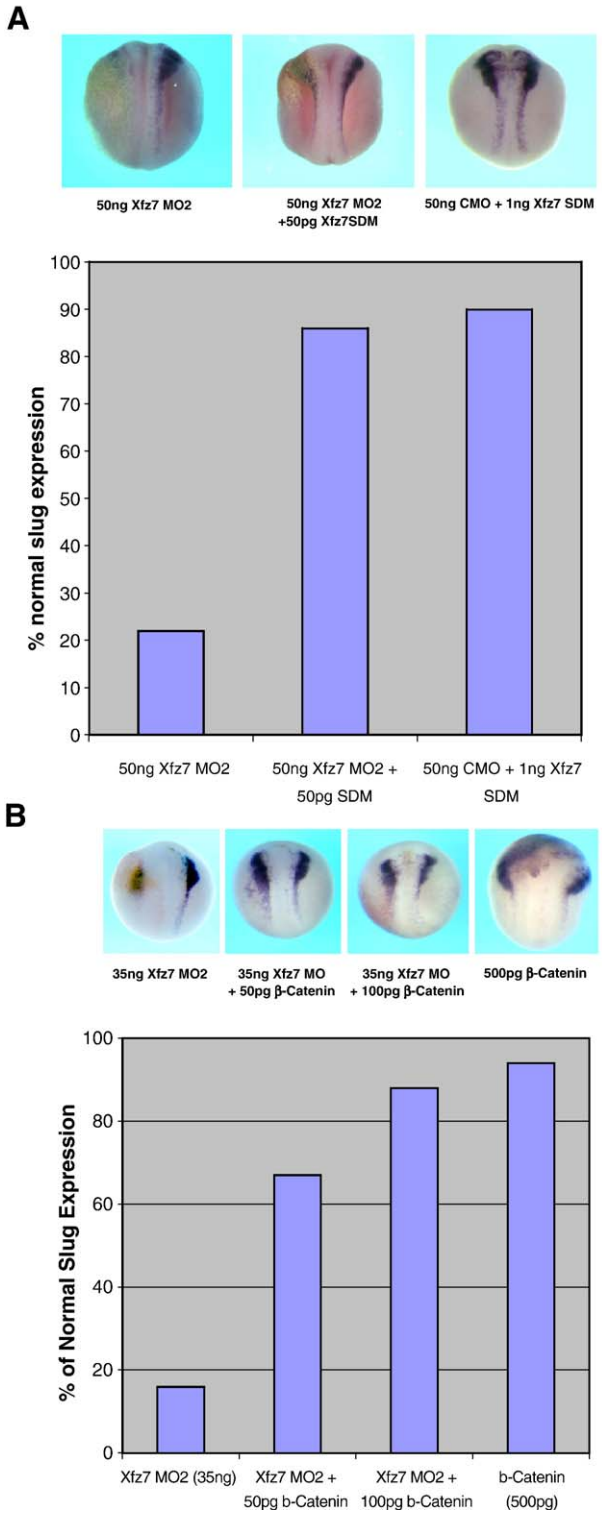

Fig. 7. Xfz7 MO-mediated downregulation of neural crest induction can be rescued by Xfz7 and β-catenin. Embryos were injected at the 2-cell stage with MO and RNA in the animal pole of one blastomere. The MOs used were Xfz7MO (A and B) and Co MO (A). RNAs used were Xfz7 SDM (A) and constitutively active β-catenin (B). (A) Xfz7 MO on its own gives 22% embryos showing normal slug expression ($n=27$). Xfz7 MO plus 50 pg Xfz7SDM gives 86% normal slug expression ($n=29$). 50 ng Co MO plus 1 ng Xfz7 SDM results in 90% of embryos having normal slug expression ($n=27$). (B) 35 ng Xfz7MO gives 16% of embryos showing normal slug expression ($n=29$). Xfz7MO plus 50 pg β-catenin and 100 pg β-catenin gives embryos showing increasingly normal slug expression (67% and 88%, respectively, $n=29$ and 34). 500 pg β-catenin on its own gives 94% of embryos with normal or increased slug expression ($n=29$). All injections contained the lineage tracer β-galactosidase (250 pg).

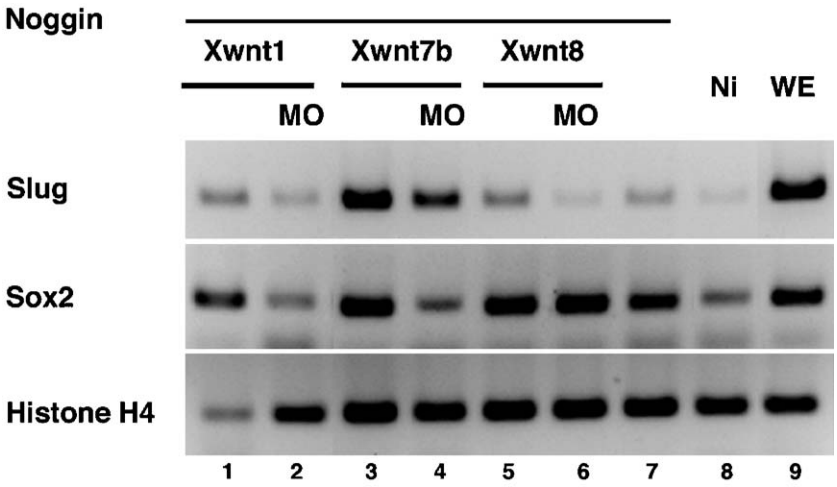

Fig. 8. Xfz7 can signal neural crest induction via a number of different Wnts. In the presence of 500 pg noggin, 100 pg of Xwnt1 (lane 1), Xwnt7b (lane 3) and Xwnt8 (lane 5), all induce slug expression. When 40 ng of Xfz7MO is co-injected, the slug signal is decreased (lanes 2, 4 and 6). 'Ni' indicates animal cap from uninjected embryo and 'WE' indicates RNA made from whole embryos at stage 19.

It is unclear why Xfz7 did not induce slug in the whole embryo when it did in the animal cap assay. However, the whole embryo is a much more complex environment. It is possible that Xfz7 activation is under the control of endogenous mechanisms such as for example Wnt antagonists present in the embryo. It has recently been demonstrated in chick that in the embryonic head region Cerberus and Dickkopf help to lower the effect of Wnt signaling at the neural plate border and thus lead to formation of the placodes at the expense of the neural crest (Litsiou et al., 2005). Another antagonist could be SFRP2, which in *Xenopus* is expressed in the prospective neuroectoderm and by mid-neurula in the anterior and posterior neural plate. In the middle region of the neural plate where Xfz7 is strongly expressed (see Fig. 1Ci), SFRP2 is expressed weakly (Shin et al., 2005). It is known that expression of En-2 requires Wnt signaling (McGrew et al., 1999). The fact that Xfz7 overexpression in the animal cap assay can induce En-2 while Xfz3 does not suggests that Xfz7 plays a role in the Wnt-mediated anterior posterior patterning of the neural tube as well as in neural crest induction. It is conceivable that Xfz7 may need specific Wnt ligand expression to give the right effect or that a particular co-receptor may be important. It is known that LRP5 and LRP6 are important for canonical signaling as are heparan sulfate proteoglycans (HSPGs) such as Glypican4 (Ohkawara et al., 2003) and the EGF-CFC family member FRL1 (Tao et al., 2005). Another possibility is that the levels of Xfz7 expression could be critical for neural crest induction (see below).

A model for neural crest induction suggests that the diffusion of BMP antagonists in the ectoderm creates a gradient of BMP activity where neural crest forms at intermediate levels of BMP activity compared with low levels for the neural plate and high levels for non-neural ectoderm (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Mayor et al., 1995, 1997). BMP signaling on its own however does not lead to neural crest induction. The role of Wnt signaling in neural crest induction has been well characterized in recent years (reviewed in Yanfeng et al., 2003). In *Xenopus*, ectopic expression of Wnts and downstream components enhances neural crest induction (De Calisto et al., 2005; Deardorff et al., 2001; LaBonne and Bronner-Fraser, 1998; Luo et al., 2003; Saint-Jeannet et al., 1997). However, how the BMP signaling pathway interacts with the Wnt pathway has remained unclear. In this paper, we have shown that Xfz7 is expressed in the dorsal ectoderm during gastrulation and that Xfz7 expression can be induced by noggin. We have also shown that the level of Xfz7 in an animal cap explant assay can determine the level of slug activation (Fig. 3C). This finding raises the possibility that Xfz7 levels determined by BMP signaling are important to modulate Wnt signals from non-neural ectoderm, neural ectoderm and/or paraxial mesoderm. We propose that tissue expressing Xfz7 at a certain level is able to respond by inducing neural crest. It will be interesting in the future to determine if the levels of Xfz7 are critical for neural crest induction, how these levels are regulated by BMP signaling and which Wnts are the important factors for neural crest formation in the whole embryo.

In our experiments, we observed a clear inhibition of the neural crest markers Xslug, XfoxD3 and Xsox10 after injection of Xfz7MO or Xfz7CRD into a 2-cell stage embryo (Figs. 4 and 5) and also when injected into the dorsal ectoderm blastomere of 8- to 16-cell stage embryos (Figs. 4H, I and K). These neural crest markers have been reported to be involved in early neural crest specification (Honore et al., 2003; Mayor et al., 2000; Sasai et al., 2001). This suggests that Xfz7 might play a role during early neural crest specification. We do see convergent extension phenotypes even in some embryos injected at later stages. However, our results suggest that this is a separable effect on morphogenesis rather than fate. Expression of the pan neural marker Xsox2 as shown in Fig. 4L shows that neural induction is occurring normally. Moreover, we see an expansion of Xsox2 at the same time that we see inhibition of the neural crest markers (Figs. 5G–I). This suggests that the inhibition of neural crest induction results in change of fate from neural crest to neural. This is consistent with the early expression of Xfz7 in neural ectoderm and the neural tube later on during

development. Similar expansion of Xsox2 expression was previously reported when a dominant negative form of XfoxD3 was injected into 2-cell stage embryos and showed down-regulation of Xslug (Sasai et al., 2001) and when morpholinos to Msx1 or Xsox10 were used in similar experiments (Honore et al., 2003; Monsoro-Burq et al., 2005).

Recently, it has been reported in the chick that neural crest induction can occur during gastrulation, much earlier than previously thought (Basch et al., 2006). Pax7 expressing cells corresponding to a specified set of cells in the chicken epiblast go on to form neural crest. Interestingly, Xfz7 is strongly expressed in the pre-gastrula dorsal ectoderm unlike Xfz3 (Fig. 1A and Wheeler and Hoppler, 1999). It is therefore possible that Xfz7 may be involved in the determination of Pax7 expressing neural crest progenitors at an earlier time than previously thought.

It has been well documented that neural crest induction is partly mediated by canonical Wnt signaling. In mice, loss of Wnt1 and Wnt3a (Ikeya et al., 1997) or of β-catenin using cre-recombinase under the control of the Wnt1 promoter (Brault et al., 2001; Hari et al., 2002) leads to deficiencies in neural crest. Depletion of β-catenin (Wu et al., 2005), overexpression of dominant negative Tcf in zebrafish (Lewis et al., 2004) as well as studies using GSK3β (Saint-Jeannet et al., 1997) and dominant negative LRP6 (Tamai et al., 2000) all suggest that canonical Wnt signaling is important in early neural crest formation. We found that Xfz7MO downregulation of slug was rescued by β-catenin, strongly suggesting that Xfz7 mediates neural crest induction via the canonical Wnt pathway. Interestingly, Xfz7 signals through both canonical and non-canonical pathways in different contexts (Medina et al., 2000; Sumanas and Ekker, 2001). For example, it synergizes with Wnt5a, Xwnt8b and/or Xwnt11 to mediate dorsal/ventral patterning and mesoderm patterning in a β-catenin-dependent mechanism (Sumanas et al., 2000) and interacts with Wnt11 to signal through a non-β-catenin-dependent pathway during convergent extension and cardiogenesis (Djiane et al., 2000; Pandur et al., 2002). Recently, it has been reported that neural crest migration is controlled by the non-canonical pathway potentially through Wnt11/Xfz7 signaling (De Calisto et al., 2005). How Xfz7 can be signaling through two different pathways is at present still unknown. One suggestion might be that the two isoforms of Xfz7 found in *Xenopus* could possibly mediate canonical and non-canonical Wnt signaling respectively. This is unlikely, however, as both isoforms of Xfz7A and B can induce slug expression in the animal cap assay and affect convergent extension movements (MAE and GNW, data not shown). Interestingly, interactions between a frizzled receptor and multiple Wnt ligands have been reported in other cases. For example, Xfz8 receptor has been shown to mediate the activity of Wnt1, Wnt2c, Wnt3a, Wnt5a, Wnt7b, Wnt8 and Wnt11 (Deardorff et al., 2001). It is possible that activation with a particular ligand may confer a signal to go through one signaling pathway rather than the other. In addition, co-receptors as mentioned above may modulate signal transduction. The presence of cofactors such as Kermit (Tan et al., 2001) may also be important. In the case of frizzled7, there is plenty of evidence that Wnt11 can bind to it and that this can activate a non-canonical signaling pathway (Djiane et al., 2000) while Tao et al. (2005) suggest an interaction of Xwnt11 and Xfz7 in β-catenin-dependent dorsal/ventral axis determination. Association of Xfz7 with Xwnt8b also leads to signaling via the canonical pathway (Medina et al., 2000). We have shown that Xfz7MO in animal caps can decrease the induction of slug mediated by Xwnt1, Xwnt7b and Xwnt8, suggesting an interaction between all these Wnts and Xfz7. These particular Wnts all activate the canonical pathway, thus suggesting that early on in the formation of the neural crest Xfz7 is signaling through the canonical pathway. Later as neural crest begin to migrate, Xwnt11 signaling, suggested to be mediated by Xfz7 as shown by their relative expression patterns (De Calisto et al., 2005) and working through the non-canonical pathway, is the crucial factor. Possibly, Wnt11 outcompetes the other Wnts for binding to Xfz7, thus switching signaling to the non-canonical pathway or activates intracellular signaling cascades maybe via changes in Ca^{+} signaling, which then antagonize the canonical pathway (Maye et al., 2004). Alternatively, specific expression of particular co-receptors could also determine which pathway is utilized. What determines the switch in signaling pathways despite the use of the same receptor will be an interesting question to investigate in the future.

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