Frizzled-7 is required for *Xenopus* heart development

Muhammad Abu-Elmagd1,2,†, Joanna Mulvaney2,†,* and Grant N. Wheeler2,§

**ABSTRACT**

Wnt signalling regulates cardiogenesis during specification of heart tissue and the morphogenetic movements necessary to form the linear heart. Wnt11-mediated non-canonical signalling promotes early cardiac development whilst Wnt11-R, which is expressed later, also signals through the non-canonical pathway to promote heart development. It is unclear which Frizzled proteins mediate these interactions. Frizzled-7 (*fzd7*) is expressed during gastrulation in the mesodermal cells fated to become heart, and then in the primary heart field. This expression is complementary to the expression of *wnt11* and *wnt11-R*. We further show co-localisation of *fzd7* with other early- and late-heart-specific markers using double *in situ* hybridisation. We have used loss of function analysis to determine the role of *fzd7* during heart development. Morpholino antisense oligonucleotide-mediated knockdown of *Fzd7* results in effects on heart development, similar to that caused by *Wnt11* loss of function. Surprisingly, overexpression of dominant-negative *Fzd7* cysteine rich domain (*Fzd7 CRD*) results in a cardiac bifida phenotype, similar to the loss of *wnt11-R* phenotype. Overexpression of *Fzd7* and activation of non-canonical wnt signalling can rescue the effect of *Fzd7 CRD*. We propose that *Fzd7* has an important role during *Xenopus* heart development.

**KEY WORDS:** *Xenopus laevis*, Cardiogenesis, Wnt signalling, Fzd7

**INTRODUCTION**

During embryogenesis, the heart is one of the first organs to form. Development of the heart includes specification of cardiac progenitors and formation of the linear heart tube by cell migration and morphogenetic movements (Mohun et al., 2000). In *Xenopus*, the heart begins to form during early gastrula stages when the cardiac progenitors arise in the dorsolateral mesoderm. Cell movements during gastrulation result in the dorso-anterior translocation of these regions and subsequent ventral migration during neurulation. The heart progenitors, which comprise cells fated to become primary or secondary heart field, form a linear heart tube at the ventral midline before looping and remodelling to form the beating heart (Kriegmair et al., 2013). Understanding the processes underlying heart development and morphogenesis are important for understanding congenital heart disease.

Heart formation is controlled by many signalling pathways including wnt signalling. *Wnt6*, *wnt11*, and *wnt11-R* have all been implicated in *Xenopus* heart development (Garriock et al., 2005; Gessert et al., 2008; Lavery et al., 2008a; Pandur et al., 2002). Wnt antagonists such as Dickkopf-1, Crescent and Sfrp1 have also been reported to control early heart formation (David et al., 2008; Foley and Mercola, 2005; Gibb et al., 2013; Marvin et al., 2001; Schneider and Mercola, 2001). Little is known however about which Frizzled proteins mediate these signals. Frizzled-7 (*fzd7*) has been well characterised in *Xenopus laevis* and other species. It has been shown to be involved in numerous developmental processes as well as being shown to be active in several forms of cancer (Huang and Klein, 2004; Liu et al., 2016; Schiffgens et al., 2016; Xu et al., 2016). *Fzd7* has been demonstrated to interact with several wnts including Wnt5a (animal cap elongation assays), Wnt6 (in somite development), Wnt8 (co-immunoprecipitation assays, *Xenopus* axis duplication) and Wnt11 (gastrulation movements, neural crest development) (Hsieh et al., 1999; Linker et al., 2005; Medina et al., 2000; Medina and Steinbeisser, 2000; Umbhauer et al., 2000; Witzel et al., 2006). It has also been shown to genetically interact with the coreceptors ror2 and ryk (Hikasa et al., 2002; Kim et al., 2008).

*Xenopus fzd7* has been implicated in gastrulation movements, tissue separation, and neural crest induction (Abu-Elmagd et al., 2006; Djiane et al., 2000; Wheeler et al., 2000; Winklbauer et al., 2001). We have previously shown *fzd7* to be expressed in the cardiac region throughout development (Wheeler and Hoppler, 1999). It has also been shown that specific depletion of *fzd7* function in *Xenopus* foregut leads to impaired cardiac morphogenesis, but has no effect on heart specification (Zhang et al., 2013). Here, we further characterise its expression relative to known heart markers, and then use whole-embryo experiments to show that *fzd7* is required for heart formation during early embryonic development.

**RESULTS**

**fzd7 expression overlaps with early heart markers**

Expression pattern analysis shows *Xenopus fzd7* is expressed in the heart-forming regions throughout development (Wheeler and Hoppler, 1999). At stage 10.5 *fzd7* is expressed in the dorsal mesoderm from which cardiac tissue originates (Wheeler and Hoppler, 1999) (Fig. 1A). As development progresses, *fzd7* expression at stage 25 is maintained in the presumptive cardiac mesoderm as it migrates dorso-laterally to the ventral midline (Fig. 1C-Cii). By stage 29, *fzd7* is expressed throughout the cardiac crescent in the cardiac mesoderm (Fig. 1E,Ei). *fzd7* expression correlates with that of *wnt11* (Fig. 1B, stage 10.5) where expression of both genes seem to be complementary in the presumptive heart region in the dorsal side of the embryo. *fzd7* expression also correlates to that of *wnt11-R* (Fig. 1D-Dii,F,Fi, stages 25 and 29, respectively) where it is expressed in the anterior endoderm at stage 25 when *fzd7* is expressed in the heart field. By stage 29, the expression of *fzd7* and *wnt11-R* overlaps (Fig. 1E-Fi). As the heart

Received 17 May 2017; Accepted 31 October 2017


This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

G.N.W., 0000-0002-4335-8577

†These authors contributed equally to this work

‡Author for correspondence (grant.wheeler@uea.ac.uk)

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Received 17 May 2017; Accepted 31 October 2017


This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.
**fzd7 is required for heart induction or specification**

Microinjection into *Xenopus* embryo dorsal blastomeres at the 4- or 8-cell stage targets prospective mesoderm including cardiac tissue. In order to test the role of *fzd7* in heart development, we inhibited its function by injecting either *fzd7* morpholino (*fzd7* MO) or its dominant-negative form expressing only the extracellular domain (cysteine rich domain, *fzd7* CRD), which would disrupt *fzd7* mediated signalling (Abu-Elmagd et al., 2006). Microinjection of *fzd7* MO into the dorsal blastomeres of 4- or 8-cell embryos leads to a reduction of both early cardiac marker *nkx2-5* (Fig. 3B-Bii) and later cardiac marker *tnnic* expression (Fig. 3E-Eii). Adding increasing amounts of *fzd7* MO leads to a progressively more severe phenotype with a greater number of embryos affected (Fig. 3C). *In situ* hybridisation for *nkx2-2.5* and *tnnic* show embryos with mild convergent extension phenotypes (Fig. 3B,E), but a severe decrease in cardiac gene expression (Fig. 3Bi,Ei) while control morpholino (CMO) show normal heart (Fig. 3A,Ai,D,Di). Some embryos also showed anterior defects (data not shown). Sections through the cardiac region showed not only a decrease of *nkx2-2.5* and *tnnic* expression, but an absence of recognisable heart structures (Fig. 3Bi,Eii) compared to CMO (Fig. 3Aii,Dii). The number of embryos injected with *fzd7* MO which showed heart and/or convergent extension and anterior defects are shown in Table S1.

Overexpression of *fzd7* full-length (*fzd7* FL) results in severe convergent extension defects, but no cardiac phenotype (Fig. S1A,B). Knockdown with *fzd7* MO can also cause a mild convergent extension phenotype and anterior defects (Abu-Elmagd et al., 2006). In order to test whether this cardiac effect is specific to *fzd7*, we rescued the *fzd7* MO cardiac phenotype with *fzd7* FL that has been mutated to not bind the *fzd7* MO (*fzd7* SDM, as described in Abu-Elmagd et al., 2006). Titrating increasing amounts of *fzd7* SDM capped RNA from 250 pg to 1 ng results in a modest rescue of the cardiac phenotype (Fig. 3F, Table S2), thus showing that *fzd7* is required for normal heart development.

Interestingly, injecting *fzd7* FL at 8-cell stage embryos shows detectable expression of *tnnic* and *nkx2-5*, despite some of these embryos showing severe convergent extension movements phenotype (head arrows in Fig. S1A,B). This leads to the suggestion that heart phenotypes are not necessarily due to convergent extension secondary effects.

**fzd7 CRD mimics wnt11-R morpholino cardiabifida phenotype and is required for non-canonical signalling**

To further look at the effect of inhibiting *fzd7* function, we took a dominant-negative approach using *fzd7* CRD. Surprisingly, this did not give a similar result to the MO knockdown. Instead, increasing amounts of *fzd7* CRD results in a dose-dependent increase in frequency and severity of cardiabifida. This was very similar to the phenotype seen for *wnt-11R* knockdown (Garriock et al., 2005). Embryos with very mild convergent extension movement defects displayed a severe cardiabifida phenotype as shown by *tnnic* expression overlaps with that of *nkx2-5* (Fig. 2B-Bii), *tnnic* (Fig. 2C-Cii) and *gata6* (Fig. 2D-Dii) in the forming heart. Interestingly, none of these markers are seen in the pericardium except for *fzd7* (Fig. 2Aii,Bii,Cii,Dii).

Fig. 1. Endogenous expression of *fzd7* in *Xenopus* heart and relative to wnt11 and wnt11-R expression. (A,B) Stage 10.5 (mid-gastrula) *fzd7* and wnt11 expression detected at the dorsal side of the embryo and appear complementary in the presumptive heart region. (C-D) *fzd7* and wnt11-R expression at stage 25. *fzd7* is seen in the heart field and wnt11-R in the anterior endoderm. *fzd7* and wnt11-R expression are complementary in the heart region (Cii,Dii). (E,F) Stage 29 embryos with *fzd7* and wnt11-R expression in the heart field, hf, heart field; ae, anterior endoderm. Magnification 20×.

continues to form, *fzd7* is strongly expressed in the lateral plates of mesoderm, cardiac mesoderm, myocardium, and over time, is restricted to the pericardium (Wheeler and Hoppler, 1999) (Fig. 2A, Aii, Bii, Cii, Dii). Using double *in situ* hybridisation, we analysed *fzd7* expression in correlation to that of early heart markers including *nkx2-5*, *troponin-ic* (*tnnic*) and *gata6*, which are all known to be required for *Xenopus* cardiogenesis (Afouda and Hoppler, 2011; Afouda et al., 2008; Drysdale et al., 1994; Flaherty and Dawn, 2008; Fu et al., 1998; Garriock et al., 2005; Jiang and Evans, 1996; Martin et al., 2010). *fzd7* expression overlaps with that of *nkx2-5* (Fig. 2B-Bii), *tnnic* (Fig. 2C-Cii) and *gata6* (Fig. 2D-Dii) in the forming heart. Interestingly, none of these markers are seen in the pericardium except for *fzd7* (Fig. 2Aii,Bii,Cii,Dii).
Control embryos showed normal expression of *tnnic* (Fig. 4A-Aii) and *nkx2-5* (Fig. 4F,Fi). These results suggest that the cardia bifida phenotype is not a secondary effect of the convergent extension defect. Overexpression of *fzd7* FL gives a severe convergent extension phenotype but no cardiac phenotype (Fig. S1A,B). Embryos with cardia bifida were unable to recover and form a normal heart when incubated up to stage 41 (n=23, data not shown). Embryos injected with a dominant-negative form of *fzd3* (*fzd3* CRD) into the dorsal blastomeres at 4-cell stage did not show cardia bifida (n= 27, Fig. 4F,Fi) indicating that the cardia bifida phenotype is specific to *fzd7* CRD. Furthermore, this phenotypic specificity to *fzd7* CRD was confirmed by rescuing the cardia bifida with *fzd7* FL-capped RNA (Fig. 5A-D,F).

It has been previously reported that a Jun N-terminal kinases (Jun) inhibitor phenocopies the *wnt11-R* cardiac phenotype of effects on cardiac morphogenesis and heart tube fusion, suggesting signalling through the non-canonical pathway (Garriock et al., 2005; Gessert et al., 2008). We therefore determined to rescue the *fzd7* CRD phenotype with *dvl1* Delta-N (*dvl1*ΔN)-capped RNA. *Dvl1*ΔN-capped RNA can rescue *fzd7* CRD (Fig. 5E,Ei,G,Gi; Table S3), suggesting that *fzd7* is required for non-canonical Wnt signalling during heart development.

**Fig. 2.** *fzd7* expression coincides with expression of the early heart markers *nkx2-5, tnnic* and *gata6*. (A-Aii) Lateral view of *Xenopus laevis* embryos at stage 31 showing *fzd7* expression detected in red and co-localised by double in situ hybridisation with other heart markers in dark blue including *nkx2-5* (B-Bii), *tnnic* (C-Cii) and *gata6* (D-Dii). (Ai,Bi,Ci,Di) Magnified lateral view of the same embryos in A, B, C and D, respectively. (Aii,Bii,Cii,Dii) Cross sections through the heart region of the embryos in A, B, C and D, respectively. *fzd7* is expressed in the myocardium and pericardium (Ai) and in other structures including neural crest, eye, pronephric duct and tail bud. *fzd7* expression shows a high degree of overlapping with the heart markers in the myocardium but not in the pericardium (Bi,Ci,Di). h, heart; c, cement gland; e, eye; nc, neural crest; pnd, pronephric duct; tb, tail bud; mc, myocardium; lpm, lateral plate of mesoderm. Magnification: 20× in A, B, C and D; 30× in Ai, Bi, Ci and Di; 200× in Aii, Bii, Cii and Dii. (Fig. 4B-Bii,C) and *nkx2-5* (Fig. 4G,Gi) expression. Control embryos showed normal expression of *tnnic* (Fig. 4A-Aii) and *nkx2-5* (Fig. 4F,Fi). These results suggest that the cardia bifida phenotype is not a secondary effect of the convergent extension defect. Overexpression of *fzd7* FL gives a severe convergent extension phenotype but no cardiac phenotype (Fig. S1A,B).

Embryos with cardia bifida were unable to recover and form a normal heart when incubated up to stage 41 (n=23, data not shown). Embryos injected with a dominant-negative form of *fzd3* (*fzd3* CRD) into the dorsal blastomeres at 4-cell stage did not show cardia bifida (n= 27, Fig. 4F,Fi) indicating that the cardia bifida phenotype is specific to *fzd7* CRD. Furthermore, this phenotypic specificity to *fzd7* CRD was confirmed by rescuing the cardia bifida with *fzd7* FL-capped RNA (Fig. 5A-D,F).

It has been previously reported that a Jun N-terminal kinases (Jun) inhibitor phenocopies the *wnt11-R* cardiac phenotype of effects on cardiac morphogenesis and heart tube fusion, suggesting signalling through the non-canonical pathway (Garriock et al., 2005; Gessert et al., 2008). We therefore determined to rescue the *fzd7* CRD phenotype with dishevelled1-Delta-N (*dvl1*ΔN)-capped RNA. *Dvl1*ΔN-capped RNA can rescue *fzd7* CRD (Fig. 5E,Ei,G,Gi; Table S3), suggesting that *fzd7* is required for non-canonical Wnt signalling during heart development.

**DISCUSSION**

Wnt signalling through the canonical and non-canonical pathways has been implicated in many aspects of heart development (Gessert and Kuhl, 2010; Ruiz-Villalba et al., 2016). How the Wnt signals that arise from both non-cardiogenic and cardiogenic tissue are integrated into heart development is less well understood. Frizzled proteins are only a part of the increasingly complicated Wnt-receptor complex found at the cell membrane, which can also include Lrp5/6, Ror2, Ryk and Kremen (Bryja et al., 2009; Korol et al., 2008; Mazzotta et al., 2016; van Wijk et al., 2009); however, Frizzled proteins are critical components of the Wnt receptor complex and so understanding their role in heart development is necessary to fully understand the
signalling involved. We have previously shown that fzd7 is expressed throughout heart development, and in this study, we show that it is functionally required in both early and late heart development. Morpholino knockdown of fzd7 leads to effects on heart development, including in some cases a complete loss of heart (Fig. 3). Overexpression of fzd7 gives rise to convergent extension defects as previously reported (Abu-Elmagd et al., 2006; Sumanas and Ekker, 2001; Winklbauer et al., 2001), but does not affect heart development. We can rescue the fzd7 MO phenotype by co-injecting site-directed mutagenized full-length fzd7 (Fig. 3). These results suggest that fzd7 is required for initial heart development, though we cannot exclude the possibility that it may also be playing a more general role in dorsoventral mesoderm patterning. Fzd7 could be interacting with Wnt11 (Kim et al., 2008; Tao et al., 2005; Witzel et al., 2006), or another wnt ligand such as Wnt3a (Mazzotta et al., 2016), Wnt6 (Gibb et al., 2013; Lavery et al., 2008a, b) or Wnt8c (Ruiz-Villalba et al., 2016; Schneider and Mercola, 2001) during these stages of development.

As suggested, it is possible that the fzd7 morphant cardiac phenotype is a secondary effect of failures in mesoderm specification, patterning, gastrulation, axis formation and tissue separation. We have made efforts to inject embryos at the 4- and 8-cell stages to give as small a convergent extension phenotype as possible to generate normal-looking embryos but with clear heart phenotypes. The results suggest that the effect of fzd7 during early heart development is not secondary to convergent extension defects or mesoderm development, however, this cannot be ruled out completely (Fig. 3).

An interesting feature of the loss-of-function analysis using fzd7 Morpholino and a dominant-negative fzd7 (fzd7 CRD), is that they give different cardiac phenotypes. fzd7 morphants have anterior defects, convergent extension defects and reduction in nkx2-5 expression; whereas fzd7 CRD-capped RNA injections result in embryos with convergent extension defects and cardia bifida, but no head defects or loss of cardiac markers. Interestingly, it has been shown that the only way to replicate the anterior defect phenotype with a fzd7 CRD construct is to inject the capped RNA into oocytes.
This could be because the relevant signalling event has been completed by time the product of mRNA injected at the 4- or 8-cell stage has been generated. It is possible that if we injected oocytes with \textit{fzd7} CRD then we might find embryos showing loss of the heart. Another possibility is that the Morpholino is able to disrupt all Wnt signalling through \textit{fzd7} by preventing translation of Fzd7 protein, but \textit{fzd7} CRD only disrupts non-canonical signalling in this context. The requirement for co-receptors in canonical signalling may allow the CRD to interact with endogenous \textit{fzd7} and any Lrps present allowing the receptor complex aggregates to form. In addition to this, it has been shown to be possible to activate canonical Wnt signalling using CRD constructs (Carron et al., 2003). Perhaps canonical Wnt signalling mediated by \textit{fzd7} early on during development is allowed to proceed by the Fzd7 CRD, but then when \textit{fzd7} switches to mediate non-canonical signalling, the CRD starts to behave as a dominant-negative. Other possibilities are that the Morpholino may have a broader specificity than thought or that the injected RNA of the \textit{fzd7} CRD construct may not be very stable, and thus only provide a short term effect compared to the Morpholino. These possibilities remain to be tested further.

The \textit{fzd7} CRD phenotype is very similar to the \textit{wnt11-R} Morpholino phenotype (Garriock et al., 2005). It has previously been shown that DM-GRASP/alcam expression lies downstream of \textit{wnt11-R} signalling and that DM-GRASP/alcam can mediate non-canonical wnt signalling effects on morphogenetic movements involved in the developing heart. The DM-GRASP/alcam Morpholino phenotype is also similar to the \textit{fzd7} CRD phenotype in that they both lead to a cardia bifida-like phenotype and a thickening of the myocardium. This suggests \textit{fzd7} could be mediating the \textit{wnt11-R} control of DM-GRASP/alcam expression. This needs to be investigated further.
Ruiz-Villalba et al. (2016) suggest a model where periodic switching between proliferation and differentiation within the developing heart is mediated by the periodic and reciprocal activity of the canonical and non-canonical wnt pathways. \textit{fzd7} could be playing a crucial role in this process depending upon the Wnts and other receptors expressed at specific times.

In conclusion, we have shown \textit{fzd7} to be involved in heart development. Further investigation is required to determine the specific wnt(s) it is interacting with at different stages of heart development.

MATERIALS AND METHODS

Embryo manipulation

All experiments were performed in compliance with the relevant laws and institutional guidelines at the University of East Anglia. The research was approved by the local ethical review committee according to UK Home Office regulations. \textit{Xenopus laevis} embryos were obtained as previously described (Harrison et al., 2004). Staging of the embryos was carried out according to the normal timetable of Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryos at the required stages were fixed in MEMFA, washed in PBS, dehydrated in ascending grades of Methanol/PBS, then stored in 100% MeOH at −20°C until processing for single or double \textit{in situ} hybridisation.

\textbf{Fig. 5. Activation of non-canonical wnt signalling rescues \textit{fzd7} CRD-induced cardia bifida.} (A,Ai) Wild-type control embryos showing normal \textit{tnnic} expression in the heart. (B,Bi) \textit{fzd7} full-length (\textit{fzd7} FL) overexpression (500 pg) injected into the dorsal blastomeres (DB) at the 4-cell stage show normal heart expression of \textit{tnnic} despite suffering a severe extension movement defect. (C) Embryos injected with 500 pg \textit{fzd7} CRD show cardia bifida phenotype, note that embryos have normal to mild convergent extension defects. (D) Rescue of the \textit{fzd7} CRD (250 pg) cardia bifida phenotype with 250 pg \textit{fzd7} FL, embryos show normal morphology as well as normal \textit{tnnic} expression. (F) Graph of \textit{fzd7} CRD cardia bifida phenotype rescue with \textit{fzd7} FL. (E,Ei) Rescue of \textit{fzd7} CRD (500 pg) cardia bifida phenotype with \textit{dishevelled1-Delta-N} (Dvl1\textit{ΔN}, 1.25 ng) indicating that \textit{fzd7} is required for the non-canonical signalling in the heart. (G) Graph of \textit{fzd7} CRD cardia bifida phenotype rescue with \textit{dvl1}\textit{ΔN}, panel Gi is the key for the cardia bifida phenotype scoring in G. Magnification 20×.

\textbf{Constructs}

\textit{fzd7} full-length (\textit{fzd7} FL) and dominant-negative form \textit{fzd7}-cysteine rich domain (\textit{fzd7} CRD) were sub-cloned into pCS2+ at \textit{ClaI}–\textit{XhoI} restriction sites as described in Wheeler et al. (2000). \textit{fzd7} MO titration by RNA in the rescue experiments was avoided by creating a site-directed mutagenesis construct of the full-coding sequence of \textit{fzd7} (\textit{fzd7} SDM) as described in Abu-Elmagd et al. (2006). \textit{fzd3} full-length (\textit{fzd3} FL) and \textit{fzd3} CRD were kind gifts from Peter Klein (University of Pennsylvania). \textit{Dishevelled} construct (Dvl1\textit{ΔN}) was a gift from Roberto Mayor (University College, London) (De Calisto et al., 2005).

\textbf{In vitro capped mRNA synthesis and embryo microinjections}

All capped mRNAs of all genes used for RNA injections were prepared according to the manufacturer’s instructions using the SP6 mMessage mMachine Ambion kit (Invitrogen:\textsuperscript{TM} AM1340). Anti-sense oligonucleotides, morpholinos (MOs), were obtained and designed by Gene Tools (www.gene-tools.com, Oregon, USA) using the reported sequence for the control morpholino (CMO) (5′-CCCTTACCTCAGTTCAATTTTA TA-3′) and \textit{fzd7} MO (5′-GCCGAGTGGACGAAATCGGCTGA-3′).
was linearised with XbaI and transcribed by T7; nks2-5 was linearised with BamHI and transcribed with T7; tropomin-IC (tmic) was linearised with Xhol and transcribed with T3; gata6 was linearised with XbaI and transcribed with T7. Promega probe synthesis manufacturing instructions were followed with fzd7 probe labelled with Fluorescence-substituted nucleotide (Fl-UTP) and for other heart makers labelled with DIG-substituted nucleotide. Each RNA probe was added to 10 ml hybridisation buffer and stored at −20°C for in situ hybridisation. Single (Harland, 1991) or double (Knecht et al., 1995) in situ hybridisation was carried out as previously described (Abu-Elmagd et al., 2006). Anti-Fu, Yan, W., Mohun, T. J. and Evans, S. M. (1998). Vertebrate Imn homologues Xnkx2-2 and Xnkx2-5 are required for heart formation in a functionally redundant manner. Development 125, 4439-4449.


Supplemental Tables and Figures

Table S1:

<table>
<thead>
<tr>
<th>fzd7 MO Injections</th>
<th>Dose (ng)</th>
<th>Total No. of Embryos</th>
<th>Anterior Defects</th>
<th>Reduced Heart</th>
<th>Anterior Defects and reduced Heart</th>
<th>Normal Morphology and reduced Heart</th>
<th>% Anterior Defects</th>
<th>% Reduced Heart</th>
<th>% Anterior Defects and reduced Heart</th>
<th>% Normal Morphology and reduced Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB</td>
<td>20</td>
<td>40</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>20</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>VB</td>
<td>20</td>
<td>26</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DB</td>
<td>40</td>
<td>43</td>
<td>15</td>
<td>19</td>
<td>15</td>
<td>4</td>
<td>35</td>
<td>44</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>VB</td>
<td>40</td>
<td>47</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DB</td>
<td>60</td>
<td>51</td>
<td>49</td>
<td>36</td>
<td>34</td>
<td>2</td>
<td>96</td>
<td>71</td>
<td>67</td>
<td>4</td>
</tr>
<tr>
<td>VB</td>
<td>60</td>
<td>66</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DB</td>
<td>70</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>VB</td>
<td>70</td>
<td>30</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

fzd7 Morpholino dose response. Increasing amounts of fzd7 MO were injected at the 4 cell stage into both blastomeres of the dorsal side (DB) of the embryo and ventral side (VB) as a control. Observed phenotypes included a range of convergent extension phenotypes from severe to mild, varying degrees of anterior defects and a reduction of n"kx2-5 or tnnic expression.
### Table S2:

<table>
<thead>
<tr>
<th>Embryo injections</th>
<th>Total No. of Embryos</th>
<th>No Heart</th>
<th>Reduced Heart</th>
<th>Normal Heart</th>
<th>% No Heart</th>
<th>% Reduced Heart</th>
<th>% Normal Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-injected control</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1ng fzd7 SDM 2x DB</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1ng fzd7 SDM 2x VB</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>60ng fzd7 MO 2x DB</td>
<td>52</td>
<td>26</td>
<td>15</td>
<td>11</td>
<td>50</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>60ng fzd7 MO 2x VB</td>
<td>50</td>
<td>1</td>
<td>3</td>
<td>46</td>
<td>2</td>
<td>6</td>
<td>92</td>
</tr>
<tr>
<td>60ng fzd7 MO + 250pg lacZ 2x DB</td>
<td>93</td>
<td>31</td>
<td>41</td>
<td>21</td>
<td>33</td>
<td>44</td>
<td>23</td>
</tr>
<tr>
<td>60ng fzd7 MO + 250pg lacZ 2x VB</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>60ng fzd7 MO + 500pg lacZ 2x DB</td>
<td>101</td>
<td>40</td>
<td>33</td>
<td>28</td>
<td>40</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>60ng fzd7 MO + 500pg lacZ 2x VB</td>
<td>89</td>
<td>3</td>
<td>0</td>
<td>86</td>
<td>3</td>
<td>0</td>
<td>97</td>
</tr>
<tr>
<td>60ng fzd7 MO + 750pg lacZ 2x DB</td>
<td>101</td>
<td>27</td>
<td>53</td>
<td>21</td>
<td>27</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>60ng fzd7 MO + 750pg lacZ 2x VB</td>
<td>74</td>
<td>0</td>
<td>0</td>
<td>74</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>60ng fzd7 MO + 1ng lacZ 2x DB</td>
<td>115</td>
<td>34</td>
<td>49</td>
<td>32</td>
<td>29</td>
<td>43</td>
<td>28</td>
</tr>
<tr>
<td>60ng fzd7 MO + 1ng lacZ 2x VB</td>
<td>75</td>
<td>0</td>
<td>1</td>
<td>74</td>
<td>0</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>60ng fzd7 MO + 250pg fzd7 SDM 2x DB</td>
<td>52</td>
<td>17</td>
<td>26</td>
<td>9</td>
<td>33</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>60ng fzd7 MO + 250pg fzd7 SDM 2x VB</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>60ng fzd7 MO + 500pg fzd7 SDM 2x DB</td>
<td>107</td>
<td>16</td>
<td>42</td>
<td>49</td>
<td>15</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>60ng fzd7 MO + 500pg fzd7 SDM 2x VB</td>
<td>59</td>
<td>0</td>
<td>1</td>
<td>58</td>
<td>0</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>60ng fzd7 MO + 750pg fzd7 SDM 2x DB</td>
<td>100</td>
<td>14</td>
<td>40</td>
<td>46</td>
<td>14</td>
<td>40</td>
<td>46</td>
</tr>
<tr>
<td>60ng fzd7 MO + 750pg fzd7 SDM 2x VB</td>
<td>75</td>
<td>0</td>
<td>1</td>
<td>74</td>
<td>0</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>60ng fzd7 MO + 1ng fzd7 SDM 2x DB</td>
<td>133</td>
<td>20</td>
<td>56</td>
<td>57</td>
<td>15</td>
<td>42</td>
<td>43</td>
</tr>
<tr>
<td>60ng fzd7 MO + 1ng fzd7 SDM 2x VB</td>
<td>107</td>
<td>0</td>
<td>1</td>
<td>106</td>
<td>0</td>
<td>1</td>
<td>99</td>
</tr>
</tbody>
</table>

**fzd7 MO phenotype is rescued by fzd7 SDM.** Injecting 1ng of fzd7SDM capped RNA does not give a cardiac phenotype. Coinjecting 60 ng fzd7 MO with from 250pg –1ng of lacZ capped RNA gives between 51% and 30% embryos with no heart and between 22% and 29% embryos with normal hearts. Coinjecting with fzd7 SDM capped RNA from 250pg- 1ng gives a dose responsive decrease of embryos with no heart 33% at 250pg to 15% at 1 ng and an increase in embryos with a normal heart from 18% at 250pg to 43% at 1ng. DB: dorsal blastomeres, VB: ventral blastomeres.
Table S3:

<table>
<thead>
<tr>
<th>Embryo injections</th>
<th>Total No. of Embryos</th>
<th>Severe Cardia Bifida</th>
<th>Mid. Cardia Bifida</th>
<th>Partial Cardia Bifida</th>
<th>Normal Heart</th>
<th>% Severe Cardia Bifida</th>
<th>% Mid. Cardia Bifida</th>
<th>% Partial Cardia Bifida</th>
<th>% Normal Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-injected control</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1.5ng dvl1ΔN 2x DB</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1.5ng dvl1 ΔN 2x VB</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 750pg lacZ 2x DB</td>
<td>92</td>
<td>29</td>
<td>25</td>
<td>23</td>
<td>15</td>
<td>32</td>
<td>27</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 750pg lacZ 2x VB</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1ng lacZ 2x DB</td>
<td>67</td>
<td>25</td>
<td>10</td>
<td>22</td>
<td>10</td>
<td>37</td>
<td>15</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1ng lacZ 2x VB</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.25ng lacZ 2x DB</td>
<td>81</td>
<td>37</td>
<td>18</td>
<td>17</td>
<td>9</td>
<td>46</td>
<td>22</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.25ng lacZ 2x VB</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.5ng lacZ 2x DB</td>
<td>37</td>
<td>10</td>
<td>16</td>
<td>8</td>
<td>3</td>
<td>27</td>
<td>43</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.5ng lacZ 2x VB</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 750pg dvl1ΔN 2x DB</td>
<td>89</td>
<td>21</td>
<td>23</td>
<td>30</td>
<td>15</td>
<td>24</td>
<td>26</td>
<td>33</td>
<td>17</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 750pg dvl1ΔN 2x VB</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1ng dvl1ΔN 2x DB</td>
<td>91</td>
<td>6</td>
<td>24</td>
<td>34</td>
<td>27</td>
<td>7</td>
<td>26</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1ng dvl1ΔN 2x VB</td>
<td>93</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>93</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.25ng dvl1ΔN 2x DB</td>
<td>67</td>
<td>8</td>
<td>11</td>
<td>13</td>
<td>35</td>
<td>12</td>
<td>16</td>
<td>19</td>
<td>52</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.25ng dvl1ΔN 2x VB</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.5 ng dvl1ΔN 2x DB</td>
<td>20</td>
<td>2</td>
<td>4</td>
<td>10</td>
<td>4</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>500pg fzd7 CRD + 1.5ng dvl1ΔN 2x VB</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

**fzd7 CRD is rescued by dvl1 Δ N.** Injecting 1.5 ng of dvl1ΔN capped RNA does not give a cardiac phenotype. Coinjecting 160 ng fzd7 CRD with from 750pg –1.5ng of lacZ capped RNA gives between 33% and 46% embryos with severe cardia bifida and between 17% and 10% embryos with normal hearts. Coinjecting with dvl1ΔN capped RNA from 750pg- 1.5ng gives a dose responsive decrease of embryos with severe cardia bifida 25% at 750pg to 7% at 1.25ng and an increase in embryos with a normal heart from 18% at 750pg to 44% at 1.25ng. DB: dorsal blastomeres, VB: ventral blastomeres.
Cardiac development is independent on the convergent extension movement defects caused by overexpression of \textit{fzd7}. (A, B). \textit{fzd7} full length (250pg) injected into the dorsal blastomeres at 8 cell stage and incubated till stage-32 showing detectable \textit{tn nic} (A) and \textit{n kx2-5} (B) expression in both normal embryos and those with convergent extension movement defects (arrow heads in A and B).