Developmental Biology 329 (2009) 327–337

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

XsFRP5 modulates endodermal organogenesis in *Xenopus laevis*

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ARTICLE INFO

Article history:
Received for publication 27 August 2008
Revised 5 March 2009
Accepted 5 March 2009
Available online 12 March 2009

Keywords:
sFRP5
Wnt2b
Organogenesis
Xenopus

ABSTRACT

Canonical Wnt signalling is known to be involved in the regulation of differentiation and proliferation in the context of endodermal organogenesis. Wnt mediated β-catenin activation is understood to be modulated by secreted Frizzled-related proteins, such as XsFRP5, which is dynamically expressed in the prospective liver/ventral pancreatic precursor cells during late neurula stages, becoming liver specific at tailbud stages and shifting to the posterior stomach/anterior duodenum territory during tadpole stages of *Xenopus* embryogenesis. These expression characteristics prompted us to analyse the function of XsFRP5 in the context of endodermal organogenesis. We demonstrate that XsFRP5 can form a complex with and inhibit a multitude of different Wnt ligands, including both canonical and non-canonical ones. Knockdown of XsFRP5 results in transient pancreatic hypoplasia as well as in an enlargement of the stomach. In VegT-injected animal cap explants, XsFRP5 can induce expression of exocrine but not endocrine pancreatic marker genes. Both, its expression characteristics as well as its interactions with XsFRP5, define Wnt2b as a putative target for XsFRP5 *in vivo*. Knockdown of Wnt2b results in a hypoplastic stomach as well as in hypoplasia of the pancreas. On the basis of these findings we propose that XsFRP5 exerts an early regulatory function in the specification of the ventral pancreas, as well as a late function in controlling stomach size via inhibition of Wnt signalling.

Introduction

Different signalling molecules of the Wnt class have been described to serve important regulatory functions in embryonic development; they were found to control cell specification events as well as differentiation and proliferation of individual organs (Clevers, 2006). Wnts constitute a family of secreted glycoproteins that bind to transmembrane receptors of the Frizzled class. The best characterized Wnt pathway is the so called canonical Wnt pathway that functions via nuclear accumulation of β-catenin, mainly influencing cell proliferation and cell fate (Willert and Jones, 2006). Other branches of Wnt signalling, referred to as non-canonical, do not rely on β-catenin and affect cell shape, polarity and motility (Kühl et al., 2000; Seifert and Mlodzik, 2007). A family of secreted Wnt antagonists, that share structural similarity with the extracellular cysteine-rich domain of Frizzled transmembrane receptors, are termed "secreted Frizzled-related proteins" (sFRPs). The sFRP class of proteins consists of sFRP1 to sFRP5, Sizzled, Sizzled2, Crescent and Tlc. The latter four are apparently not present in mammals, but have been identified in *Xenopus*, zebrafish and chicken. Surprisingly, Sizzled was characterized as a BMP antagonist, which does not appear to bind Wnts (Lee et al., 2006). In general, sFRPs have been proposed to act as antagonists by preventing Wnt proteins from forming functional Wnt-Frizzled complexes (Bovolenta et al., 2008; Kawano and Kypta, 2003). However, there is only limited information available on specificity and exact mode of operation for sFRPs in respect to their inhibitory function on the Wnt signalling activities; sFRPs might also be capable of agonizing Wnt action, as observed for sFRP1 at a low dose (Uren et al., 2000).

The endodermal germ layer gives rise to the gastrointestinal tract with its associated organs, such as thyroid gland, lung, liver, pancreas and gall bladder. Previous studies have revealed that Wnt signalling plays important roles in the formation and regionalization of the vertebrate endoderm. A conditional knockout of β-catenin in the embryonic endoderm changes cell fate from endoderm to precardiac mesoderm (Lickert et al., 2002). In amphibia, β-catenin signalling plays a crucial role in the establishment of the dorsoventral body axis (Moon and Kimelman, 1998). In addition to these very early functions, canonical Wnt signals were further reported to regulate anterior–posterior patterning of the primitive gut tube. Overexpression of a constitutively active form of β-catenin in the murine lung resulted in ectopic intestinal marker gene expression (Okubo and Hogan, 2004). The Barx1-mediated secretion of the Wnt antagonists sFRP1 and sFRP2 is essential during specification of the stomach epithelium in the mouse (Kim et al., 2005), while the double knockout of TCF1 and TCF4 led to anterior transformations at the stomach–duodenal border (Gregorieff et al., 2004). In chicken, β-catenin positively regulates proliferation of hepatic precursor cells; overexpression of β-catenin resulted in a largely expanded liver, whereas inhibition of β-catenin led to a hypoplastic liver (Suksaweang et al., 2004). In accordance with these findings, it could be shown that mesodermal Wnt2bb is

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doi:10.1016/j.ydbio.2009.03.004

essential for the specification of hepatic precursor cells in the zebrafish (Ober et al., 2006). In murine pancreas development, a bi-phasic effect of β-catenin activity on organ development was described; early embryonic overexpression led to pancreatic hypoplasia, whereas ectopic expression of β-catenin at E13.5 resulted in a strong increase in pancreas mass (Heiser et al., 2006).

In *Xenopus*, an early role for Wnt signalling in the anterior–posterior patterning of the endoderm has been revealed more recently by both inhibition as well as ectopic activation of β-catenin activity in the endoderm of gastrula stage embryos; ectopic expression of liver and pancreas marker gene expression in the posterior endoderm was achieved by inhibition, and suppression of the same genes was observed upon over-activation of canonical Wnt signalling. On the basis of these results it was proposed that endoderm patterning might be regulated in striking similarity to the anterior–posterior patterning of the central nervous system, that relies on inhibition of Wnt signalling anteriorly and higher levels posteriorly (McLin et al., 2007).

One member of the sFRP class of Wnt antagonists in *Xenopus*, namely XsFRP5, was shown to be expressed in a pattern overlapping with and extending dorsally to the field of liver precursor cells at late neurula stage; these expression characteristics are maintained to tailbud stages of development. XsFRP5 expression shifts to other endodermal tissues coincident with the posterior stomach and anterior duodenum during later development. It is however never detected in the pancreas during later stages (Pilcher and Krieg, 2002). The mouse orthologue, Sfrp5, is expressed already in the anterior visceral endoderm and later in the foregut endoderm, which will give rise to the liver (Finley et al., 2003). Nevertheless, Sfrp5 depletion does not appear to affect axis formation nor Hex expression as a molecular marker for liver development (Leaf et al., 2006); a weak effect on somitogenesis has been reported most recently in Sfrp1/Sfrp2/Sfrp5 compound knockout mice (Satoh et al., 2008).

The aim of this study was to functionally characterize XsFRP5 during gastrointestinal tract development in *Xenopus*. We report that XsFRP5 can inhibit a broad range of canonical and non-canonical Xwnt proteins. Furthermore, we reveal a regulatory function for XsFRP5 during endodermal organogenesis and propose that XsFRP5 controls specification of the ventral pancreatic anlage as well as proliferation of the stomach epithelium, at least in part via inhibition of Xwnt2b.

Materials and methods

Embryo culture and microinjection

Xenopus embryos were obtained from HCG-injected females using standard techniques and staged according to Nieuwkoop and Faber (1967). Capped mRNA for microinjection was transcribed *in vitro* (mMessage mMachine Kit, Ambion) and purified chromatographically (RNeasy, Qiagen). Enzymes used for linearization and transcription as well as the injected amounts per embryo were as follows: pCS2+/GFP (NotI; SP6; 500 pg), pCS2+/5′UTR–XsFRP5–GFP (NotI; SP6; 500 pg), pCS2+/hFz5 (NsiI; Sp6; 50 pg), pCS2+/VegT (NotI; SP6; 200 pg), pCS2+/XsFRP5 (NotI; SP6: 50, 100, 200 pg), pCS2+/XsFRP5-HA (NotI; SP6; 500 pg), pcDNA3/XsFRP2 (XhoI; T7; 200, 400 pg), pXT7/Xwnt2b (SalI; T7; 60 pg), pCS2+/MT-Xwnt2b (NotI; SP6; 500 pg), pSP64T-Xwnt3a (NcoI; SP6; 10 pg), pCS2+/MT-Xwnt3a (NotI; SP6; 500 pg), pSP64T-Xwnt4 (SalI; SP6; 5 pg), pCS2+/MT-Xwnt4 (NotI; SP6; 500 pg), pSP64T-Xwnt5a (XbaI; SP6; 5 pg), pCS2+/MT-Xwnt5a (NotI; SP6; 500 pg), pSP64T-Xwnt8 (BamHI; SP6; 10 pg), pCS2+/MT-Xwnt8 (NotI; SP6; 500 pg), pSP64T-Xwnt8b (SalI; SP6; 10 pg), pCS2+/MT-Xwnt8b (NotI; SP6; 500 pg), pCS2+/Xwnt11 (SacI; T7; 5 pg), pCS2+/MT-Xwnt11 (NotI; SP6; 500 pg), pCS2+/Xwnt11R (NotI; SP6; 5 pg), and pCS2+/MT-Xwnt11R (NotI; SP6; 500 pg). For axis duplication assays, embryos were injected marginally into one ventral blastomere at the four cell stage and nuclear LacZ mRNA was used to equilibrate the injected RNA quantities. Axis duplication assays for each different Xwnt were at least performed twice. Morpholino antisense oligonucleotides (MOs) were ordered from GeneTools and injected at the four/eight cell-stage into all four blastomeres at the vegetal pole. The Xwnt2b splice-morpholino (SpliMO) is directed against the exon3/intron3 junction and Xwnt2b-SpliMO2 is directed against intron3/exon4 junction of *Xenopus laevis* Wnt2b. Intron3 was PCR-amplified from genomic DNA, sub-cloned and sequenced. Sequences of the MOs are: XsFRP5-MO1 (5′-ATCGCATGATTTCTCCAAAAAGTGG-3′); mmMO1 (5′-ATGGCATCATATCTCGAAAAACTGG-3′); XsFRP5-MO2 (5′-TTTAACCCAAAGGCTTATCCTCCTG-3′); Xwnt2b-SpliMO (5′-CACGCTCAGCAAGACTTACCATTC-3); Xwnt2b-MO (5′-GTATTAAAATATAAGCAAAA-TGCAT-3′); Xwnt2b-SpliMO2 (5′-TTTACTGCCTGTAAGATAGGAAAAA-3′). Injected amounts were 5 ng of the XsFRP5-MO1 and mmMO, 2 ng of XsFRP5-MO2, 20 ng of Xwnt2b-SpliMO and Xwnt2b-MO as well as 10 ng Xwnt2b-SpliMO2. 150 pg nuclear LacZ mRNA was co-injected as a lineage tracer.

Microdissection of stage 40 gut tubes

Embryos were cultured until stage 40 and after 30 min prefixing in MEMFA, gut tubes were explanted as described previously (Chalmers and Slack, 1998). Explants were refixed for 15 min in MEMFA and either X-Gal stained or transferred to and stored in ethanol.

Whole-mount in situ hybridisation

The embryos and explants were stained for X-Gal activity as described (Hardcastle and Papalopulu, 2000). Whole-mount *in situ* hybridisation was performed as described previously (Harland, 1991; Hollemann et al., 1999) with Digoxigenin-UTP (Roche) labelled antisense probes. The following antisense probes were prepared as described previously: Ptf1a/p48 and XlHbox8 (Chen et al., 2004), XPDIp (Afelik et al., 2004). Enzymes used for linearization and *in vitro* transcription are indicated for: pBK-CMV-Fibrinogen (NcoI; T3), pBSK-Sox2 (EcoRI; T7), pGEMT-XsFRP5 (SphI; SP6), pCS2+/MT-Xwnt2b (EcoRI; T7), pGEMT-Xwnt5a (NcoI; SP6), and pCS2+/Xwnt11 (BamHI; T7).

pH3 staining

Control and morpholino-injected embryos were fixed in MEMFA at stage 32, stage 37/38 and stage 40 (gut tube explants), transferred to Dent's solution and stored at −20 °C for 1 week. The pH 3 assay was performed as described previously (Dent et al., 1989). As first antibody we used the rabbit anti-pH3 antibody from Biomol and as secondary antibody the goat anti-rabbit IgG alkaline phosphatase-coupled antibody from Sigma.

Vibratome sections

30 μm vibratome sections were prepared as described previously (Hollemann and Pieler, 1999) using a Leica VT1000S Vibratome.

Co-immunoprecipitation

XsFRP5 and Xwnt constructs were PCR-amplified and subcloned into pCS2+/HA or pCS2+/6xMyc, respectively. Embryos were injected animally into both blastomeres at the two cell stage and cultured until stage 10.5. Co-IP was performed as described (Djiane et al., 2000), using anti-HA IgG (MMS-101P, Covance).

Animal cap dissection and RT-PCR analysis

Xenopus embryos were injected animally into both blastomeres at the two cell stage. Animal caps were isolated at stage 8/9 and cultured

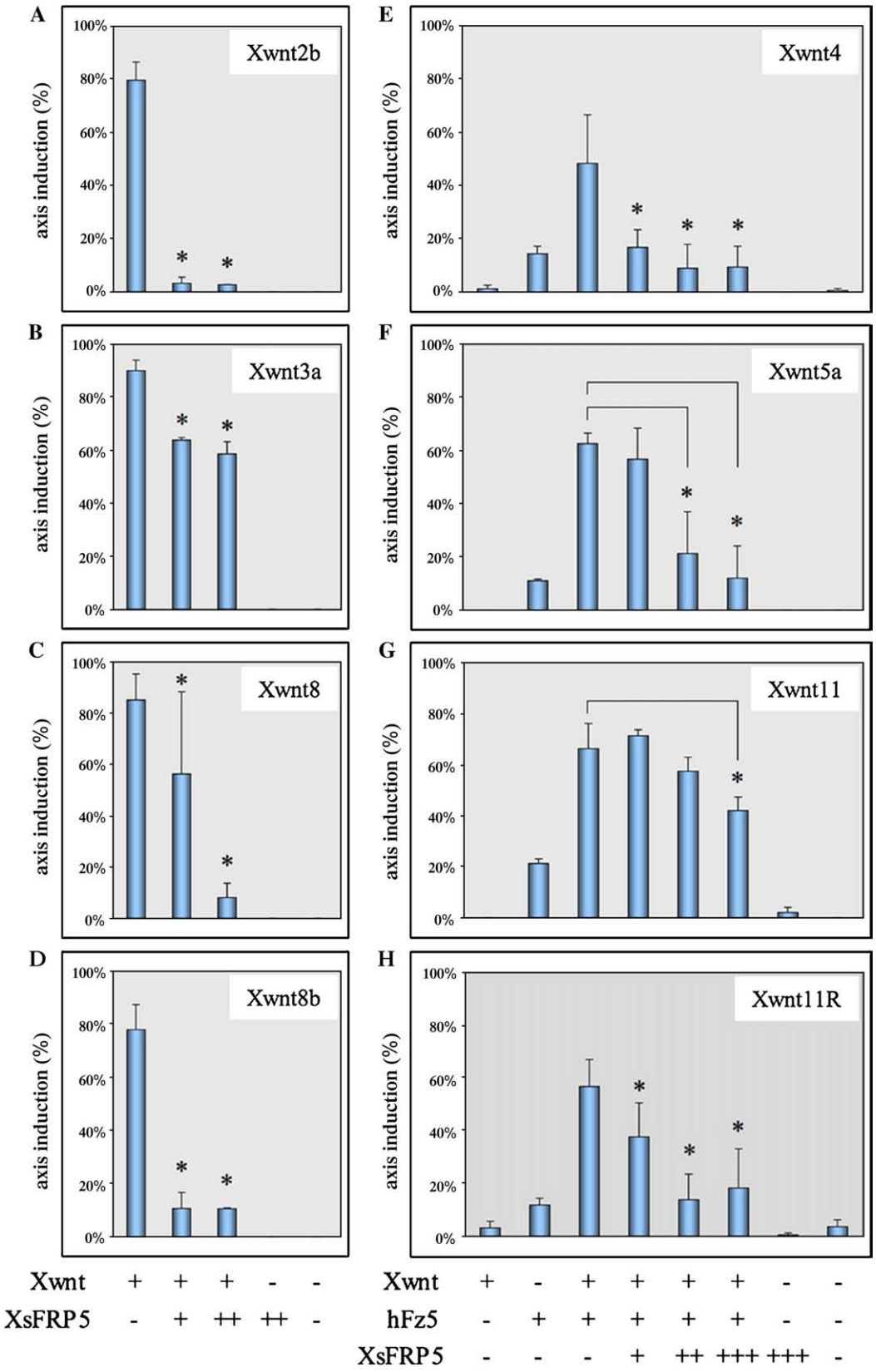

Fig. 1. XsFRP5 inhibits dorsal axis duplication induced by various canonical and non-canonical Xwnt proteins. Axis duplication was achieved by injecting 60 pg Xwnt2b (A), 10 pg Xwnt3a (B), Xwnt8 (C) and Xwnt8b (D) mRNA as well as 5 pg Xwnt4 (E), Xwnt5a (F), Xwnt11 (G) and Xwnt11R (H) mRNA into one of two ventral blastomeres at the four cell stage; 50 pg hFz5 mRNA were co-injected in (E–H). 10 (+), 50 (+) or 100 pg (++) XsFRP5 mRNA were co-injected, as indicated. Embryos were scored for dorsal axis duplication at early tailbud stage. The significance of values obtained for reduction of Wnt-mediated axis duplication was calculated using standardised normal distribution and is indicated with asterisks.

until sibling embryos had reached stage 40. Total RNA was extracted using Trizol and treated with DNaseI. All samples were controlled for genomic DNA contamination by 30 cycles of Histone H4-specific PCR without the use of reverse transcriptase. cDNA was prepared using random hexamer primers and was analysed by semi-quantitative RT-PCR for marker gene expression using the GoTaq Kit (Promega). The following primer sequences and cycle numbers were published elsewhere: Histone H4, Transthyretin, Insulin and XlHbox8 (Chen et al., 2003), Xbra (Batut et al., 2005), XPDIp (Afelik et al., 2004), XTwist (Pan et al., 2007), and Sox2 (Nitta et al., 2006). For other marker genes, the primer pairs and cycle numbers were: Ptf1a/p48 (fw: 5′-GAGAAGCGACTGTCCAAG-3′; rev: 5′-CATCAGTCCATGAG-AGAG-3′; 33 cycles), Xnr3 (fw: 5′-GACCAGGGGAAAGAGGTT-3′; rev: 5′-GGGATCAGGTTTAGCATGAG-3′; 32 cycles), XsFRP5 (fw: 5′-AGGTCCTTAAAGCAGGGAAG-3′; rev: 5′-GCGATGGTGGCACTTAAAG-3′; 34 cycles), and Xwnt2b (fw: 5′-ACTGGGAGCAAGGGTTATCTGTG-3′; rev: 5′-GACCCCGTGGCACTTAC-3′; 33 cycles).

Chemical treatment of animal cap explants

Animal cap explants were treated with 5 μM retinoic acid (RA) at stage 9 for 1 h, as described previously (Pan et al., 2007). Animal caps were treated with 1 μM BMS453 at stage 9 for 1 h in the dark. Control caps were treated with ethanol or DMSO, respectively.

Results

XsFRP5 functions as an inhibitor of canonical and non-canonical Wnt signalling

sFRPs have been proposed to function both as agonists and as antagonists of Wnt signalling activity (Bovolenta et al., 2008; Kawano and Kypta, 2003). Bovine sFRP5 has previously been reported to exert a weak inhibitory activity in *Xenopus* Wnt8 induced secondary body axis formation in *Xenopus* embryos (Chang et al., 1999). In order to analyse the activity and specificity of *Xenopus* sFRP5 more systematically, we employed four different assay systems. Firstly, we tested the effect of XsFRP5 on the ability of a number of different canonical and non-canonical *Xenopus* Wnts to induce secondary axis formation in whole embryos and, secondly, to induce canonical Wnt target gene transcription in animal cap explants. Thirdly, we analysed the morphological effects of XsFRP5 overexpression on gastrulation movements, which are known to be regulated by endogenous, non-canonical Wnt signalling, and, finally, we also tested integration of XsFRP5 into one complex with the same panel of Wnts in co-immunoprecipitation assays from microinjected *Xenopus* embryos.

Of the four different canonical Wnts analysed, two, namely Xwnt2b and Xwnt8b, were found to be significantly inhibited (more than twofold) in respect to their axis inducing activity already at the lowest dose of XsFRP5 tested (Figs. 1A, D); a third one, Xwnt8, revealed a significant response at the intermediate dose, while the fourth one, Xwnt3a, was only weakly inhibited even at the highest dose tested (Figs. 1B, C). Xnr3 gene transcription is known to be stimulated by β-catenin mediated Wnt signalling in microinjected animal cap explants (McKendry et al., 1997); in this scenario, XsFRP5 completely blocks Xwnt2b, Xwnt8 and Xwnt8b, while Xwnt3a was only moderately inhibited (Fig. 2A). Thus, there is a very good correlation of results obtained in these two first assays for the effects of XsFRP5 on canonical Wnt signalling.

The non-canonical Wnts require co-injection of hFz5 in order to elucidate their axis-inducing activities (He et al., 1997). As for the canonical Wnt ligands, multiple of the non-canonical ones were found to be inhibited significantly by XsFRP5: Xwnt4 already at the lowest dose tested, Xwnt5a and Xwnt11R at intermediate doses and Xwnt11 only moderately, even at the highest dose tested (Figs. 1E–H). The ability of XsFRP5 to inhibit non-canonical Wnts should also affect gastrulation movements, as they are known to be regulated by non-canonical Wnt signalling. Vegetal injection of XsFRP5 at the two cell

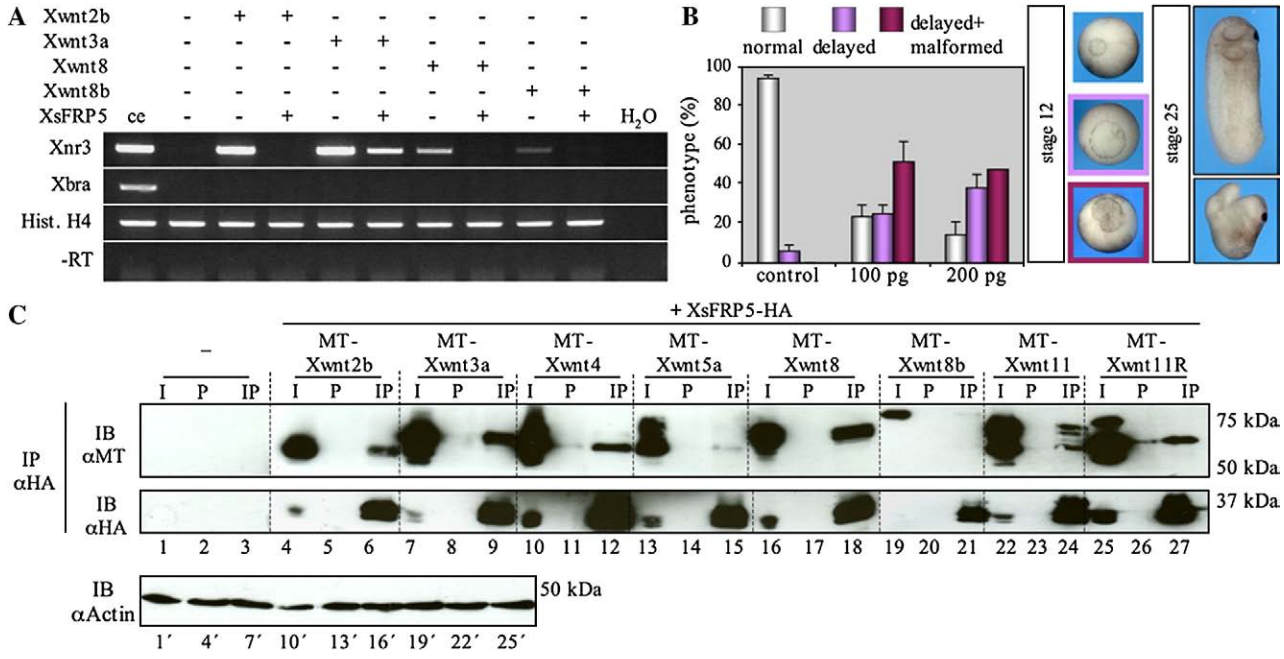

Fig. 2. XsFRP5 functions as an inhibitor of canonical and non-canonical Wnt signalling. (A) XsFRP5 can suppress Xwnt-induced Xnr3 gene expression in animal cap explants. 60 pg Xwnt2b or 10 pg Xwnt3a, Xwnt8 and Xwnt8b mRNAs were injected with or without 100 pg XsFRP5 mRNA animally into both blastomeres at the two cell stage. Animal cap explants were isolated at stage 8 and grown to the equivalent of stage 11.5. RNA preparations were analysed by semi-quantitative RT-PCR for transcriptional activation of the genes indicated. (B) Overexpression of XsFRP5 interferes with gastrulation movements. Embryos were injected vegetally into both blastomeres with 100 pg or 200 pg XsFRP5 mRNA and harvested when sibling control embryos had reached stage 12 and stage 25, respectively. (C) Xwnt proteins co-precipitate with XsFRP5. As indicated, tagged fusion constructs were injected animally at the two cell stage and embryos were harvested at gastrula stage. Immunoprecipitation was performed from embryonic extracts using an antibody directed against HA-tagged XsFRP5 and the samples were analysed by Western Blot. Actin was used as loading control for the input. I = input (10% crude extract), P = preclearing pellet, IP = immunopellet, IB = immunoblot.

stage led to delayed blastopore closure and malformed blastopore shape as compared to sibling control embryos, a phenotype that is indicative for defects in convergent extension cell movements (Fig. 2B). When such XsFRP5-injected embryos were grown to later stages of development, they exhibited a severely shortened A–P body axis; similar phenotypes have been described for other proteins that interfere with non-canonical Wnt signalling (Kühl et al., 2001; Tahinci et al., 2007; Yamanaka et al., 2002). These observations provide a clear further indication for XsFRP5 being able to inhibit non-canonical Wnt signalling in early *Xenopus* embryos. Thus, in summary, XsFRP5 can function as an inhibitor of both canonical and non-canonical Wnt signalling, and it appears to possess significantly different relative efficiencies in respect to its inhibitory effects on the eight different Wnt ligands employed in the above assays.

The inhibitory activities of XsFRP5 in the axis duplication assay could reflect complex formation with Wnt ligands and perhaps other proteins, or they could be the consequence of a more indirect mechanism. To test this hypothesis, epitope-tagged versions of various Xwnt proteins were analysed for their ability to co-immunoprecipitate from injected embryonic extracts (Fig. 2C). In most assays, the Wnt proteins gave rise to several electrophoretically distinct signals in the input samples, possibly representing the different processing events leading to the formation of biologically active signalling molecules. Xwnt2b, 3a, 4, 8, 11 and 11R were found to co-precipitate in significant quantities with XsFRP5, whereas Xwnt5a and Xwnt8b were only very weakly detected. Thus, XsFRP5 is able to form complexes including both canonical and non-canonical Wnts. This is consistent with the general conclusion drawn from the above experiments testing for biological activities. However, there are several exceptions: Xwnt8b, was efficiently inhibited in the axis duplication and animal cap assays, while it was only very weakly co-immunoprecipitated; conversely, Xwnt3a was found to be grossly complexed with XsFRP5, while being only moderately inhibited in the functional assays.

Knockdown of XsFRP5 results in a hypoplastic ventral pancreatic anlage as well as in stomach enlargement

In order to suppress XsFRP5 translation, two different antisense morpholino oligonucleotides were designed, one spanning the region of the start codon (MO1) and the second one (MO2) targeting a

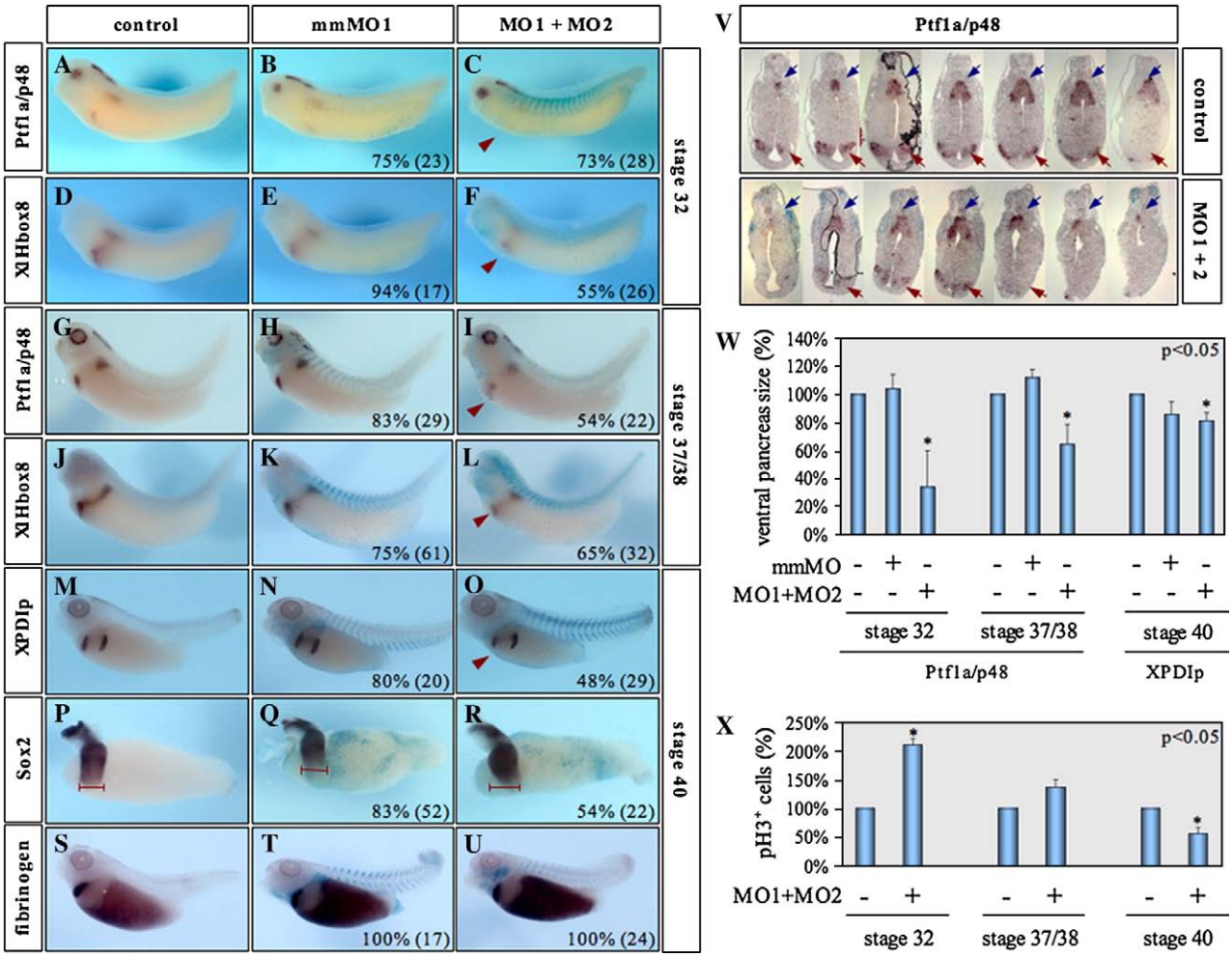

Fig. 3. Knockdown of XsFRP5 expression by morpholino injection leads to a hypoplastic ventral pancreas and stomach enlargement. (A–U) Endodermal marker gene expression analysis by whole-mount *in situ* hybridisation. 5 ng mmMO1 or a combination of 5 ng MO1 and 2 ng MO2 were injected into the vegetal pole of all four blastomeres at the four cell stage. LacZ activity was used as a lineage tracer. Whole-mount *in situ* hybridisation was performed with stage 32 (A–F), stage 37/38 (G–L) and stage 40 embryos (M–U). The red bars indicate the width of the Sox2 expression domain (P–R). The red arrowheads mark the ventral pancreatic anlage (C, F, I, L, O). (V) Vibratome sections of stage 37/38 control and MO1 + MO2-injected embryos stained for Ptf1a/p48. The blue arrows indicate the dorsal pancreatic anlage and the red arrows mark the ventral anlagen. (W) The size of the ventral pancreatic anlagen was quantified in control, mmMO- and MO1 + MO2-injected embryos stained for Ptf1a/p48 or XPDIp. The average pixel number in the controls was set to 100%. Significance was calculated using Student's *T* Test and is marked with asterisks. (X) Vibratome sections of stage 32 and stage 37/38 embryos, as well as stage 40 gut explants were quantified for proliferative cells using pH3-labelling. 10 consecutive sections of 3 embryos/explants were scored for $pH3^+$ cells in the anterior endoderm at stages 32 and 37/38, and in the stomach/pancreas/liver area of stage 40 gut explants. The mean value of $pH3^+$ cells in the controls was set to 100%. Significance was calculated using Student's *T* Test and is marked with asterisks.

sequence 50 bp upstream of the initiation codon in the XsFRP5 mRNA; a five nucleotide mismatch version of MO1 (mmMO1) served as a control (Supplementary fig. 1A). All morpholinos were tested *in vitro* and *in vivo* for their ability to block XsFRP5 protein synthesis. Both MO1 and MO2 reduced XsFRP5 protein translation *in vitro*; in combination, they completely blocked translation, while the mismatched morpholino had no significant effect (Supplementary fig. 1B). For the purpose of additional control experiments, GFP was fused to the XsFRP5–5′UTR and injected into early cleavage stages with or without the different MOs. Injection of MO1 and MO2 blocked GFP expression completely, whereas mmMO1 had no significant effect; expression of a GFP construct without morpholino binding sites was not affected by any of these manipulations (Supplementary fig. 1C). Thus, both MO1 and MO2 function specifically in the *in vitro* as well as in the *in vivo* situation.

To knockdown expression of XsFRP5 in *Xenopus* embryos, a combination of MO1 and MO2 was injected vegetally into all four blastomeres at the four/eight cell stage to target the presumptive endoderm, and resulting effects on endodermal organogenesis were monitored by *in situ* hybridisation (Figs. 3A–U). Knockdown of XsFRP5 resulted in inhibition of pancreas specification as revealed by reduced endodermal Ptf1a/p48 expression at stage 32 (Figs. 3C and W); at stage 37/38, inhibition of Ptf1a/p48 expression in the ventral pancreas persisted, while dorsal pancreas formation, as well as other Ptf1a/p48 expression domains in retina and hindbrain, were largely unaffected in XsFRP5 morphant embryos (Figs. 3I, V and W). XlHbox8, a second pancreatic precursor cell marker that is additionally expressed in duodenum and posterior stomach, similarly reveals early pancreatic hypoplasia, as well as a partial loss of expression in the duodenum (Figs. 3F and L). Even though the inhibitory effects on pancreas development upon XsFRP5 knockdown appear to be most pronounced at specification stages (stage 32), they also correlate with a smaller pancreas size in later development (stage 40), as shown by reduced XPDIp staining, marking differentiated exocrine pancreatic tissue (Figs. 3O and W). To analyse effects on stomach development, endoderm explants from stage 40 were analysed for Sox2 expression. At this stage, Sox2 marks the oesophagus, the stomach and an anterior portion of the duodenum (Fig. 3P). Loss of XsFRP5 leads to an enlargement of the stomach (Fig. 3R), as compared to the uninjected control embryos. Fibrinogen, which is expressed in liver and intestine of stage 40 embryos, remains undisturbed (Figs. 3S–U). Since organ enlargement could be the result of increased proliferation, we further compared morphant and control embryos for effects on phospho-Histone H3 (pH3) staining in the anterior endoderm of stages 32 and 37/38 embryos, as well as in the stomach/liver/duodenum territory of stage 40 endodermal explants; knockdown of XsFRP5 correlates with a significant increase in the number of proliferating cells in the anterior endoderm of stage 32 embryos (Fig. 3X). Thus, knockdown of

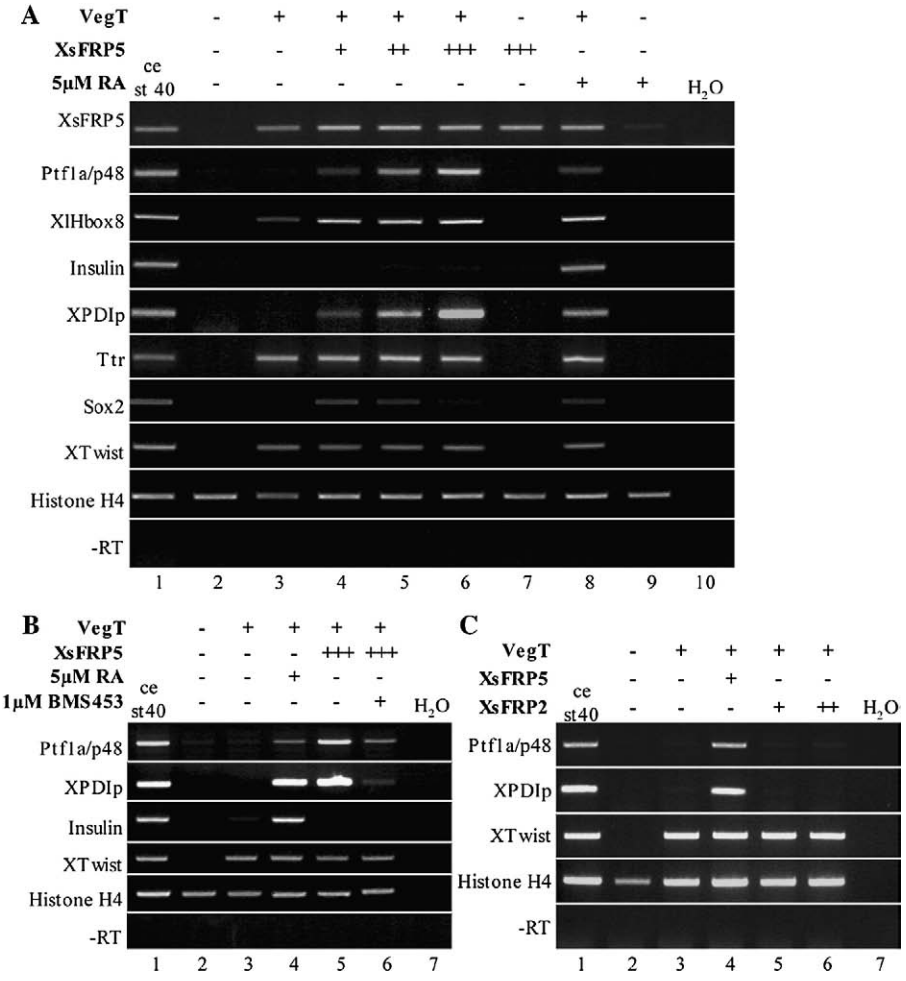

Fig. 4. XsFRP5 can substitute for retinoic acid in respect to the induction of exocrine cell differentiation in VegT injected animal caps. (A, B) Embryos were injected with 200 pg VegT and 50, 100 or 200 pg XsFRP5 mRNA animally into both blastomeres at the two cell stage and animal cap explants were isolated at stages 8 to 9. The explants were cultured until control embryos had reached stage 40 and analysed by semi-quantitative RT-PCR. Treatment with 5 µM retinoic acid or 1 µM BMS453 was performed for 1 h at stage 9. (C) XsFRP2 cannot phenocopy the XsFRP5-induced pancreatic marker gene expression in VegT injected animal cap explants. Embryos were injected with 200 pg VegT plus 200 pg XsFRP5, or plus 200 and 400 pg XsFRP2 mRNA respectively and treated as stated above.

XsFRP5 results in pancreatic hypoplasia and stomach enlargement, as well as in increased proliferative activity in the anterior endoderm during pancreas specification stages of development.

XsFRP5 can substitute the need for retinoic acid in exocrine differentiation

As we have reported in a previous study, combined VegT injection and retinoic acid (RA) treatment of animal cap explants induces both exo- and endocrine-specific pancreatic marker gene expression, while injection of VegT alone is sufficient to induce the expression of duodenal and hepatic marker genes (Chen et al., 2004; Pan et al., 2007; see also Fig. 4A, lanes 3 and 8). We observed that both naïve and VegT injected explants express a variety of different canonical and non-canonical Wnt ligands (data not shown); therefore, a possible influence of XsFRP5 on this system was analysed. Co-injecting increasing amounts of XsFRP5 along with VegT, without adding exogenous RA, led to a robust and dose-dependent induction of the pancreatic precursor cell markers Ptf1a/p48 and XlHbox8, as well as of the exocrine differentiation marker XPDIp. Insulin expression, on the other hand, could not be induced (Fig. 4A, lanes 4–6). Expression of the liver differentiation marker Ttr did not change when VegT and XsFRP5 were co-expressed, as compared to VegT injected animal cap explants. Sox2 expression was weakly induced in VegT injected caps by co-injection of low amounts of XsFRP5, whereas higher amounts of XsFRP5 abolished Sox2 expression. Thus, XsFRP5 can substitute for the requirement of retinoic acid in respect to the induction of exocrine but not of endocrine pancreatic cell differentiation.

To address the question if exocrine pancreatic marker gene activation in this system by XsFRP5 would still require downstream retinoic acid signalling, VegT/XsFRP5 co-injected animal cap explants were treated with the retinoic acid antagonist BMS453 and analysed for pancreatic marker gene expression when sibling embryos had reached organogenesis stage. Ptf1a/p48 as well as XPDIp expression was strongly reduced upon BMS453-treatment in XsFRP5/VegT co-injected animal cap explants (Fig. 4B). To further address the question if the observed induction of pancreatic marker genes in VegT-injected animal caps is specific for XsFRP5, the closely related XsFRP2 was tested in the same assay. The XsFRP5-mediated induction of Ptf1a/p48 and XPDIp expression in VegT-injected animal caps could not be phenocopied by co-injection of XsFRP2, not even at an increased concentration (Fig. 4C). Thus, in conclusion, these experiments reveal that XsFRP5 can specifically substitute for the requirement for RA in respect to the specific induction of exocrine pancreatic marker gene expression in VegT injected animal cap explants, probably acting upstream of the RA signalling pathway.

Xwnt2b is a putative target for XsFRP5 in the context of endoderm patterning

In order to identify a putative target Wnt ligand for XsFRP5 in the context of stomach/duodenum/pancreas development (endoderm patterning), we compared the spatio-temporal expression pattern of XsFRP5 with the ones of the various different Wnt proteins tested above, since putative *in vivo* Wnt targets should be expressed complementary to or overlapping with XsFRP5. Depicted in Fig. 5 are only those Xwnts, which were significantly inhibited in the axis duplication assays (Fig. 1), co-precipitated efficiently with XsFRP5 (Fig. 2C) and revealed overlapping or complementary expression domains with XsFRP5 during organogenesis stages. Consistent with the original description by Pilcher and Krieg (2002), we find XsFRP5 to be expressed in the liver diverticulum at stage 35 and in stomach/anterior duodenum at stage 40. Confirming and extending the earlier description of Xwnt2b expression (Landesman and Sokol, 1997), stage 35 embryos reveal bilateral, mesodermal staining in a narrowly defined stripe of cells lying in juxtaposition to the liver/pancreas/stomach forming endoderm (Fig. 5D); stage 40 endodermal explants exhibit strong anterior staining in the developing lung (Fig. 5F). At stage 35, Xwnt5a is expressed as published earlier (Moon et al., 1993), demarcating the oesophagus, lung and anterior stomach at stage 40 (Figs. 5G–I). In stage 35 embryos, Xwnt11R is expressed in the somites and in the first branchial arch (Garriock et al., 2005). At stage 40, we

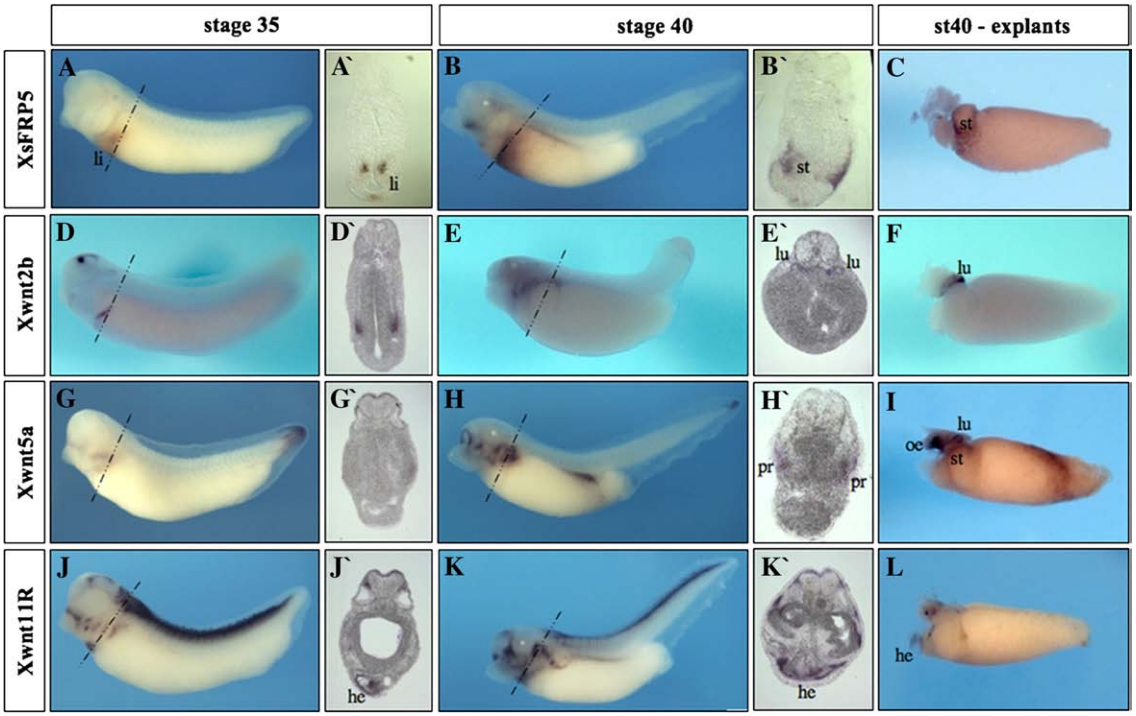

Fig. 5. Comparative expression analysis of XsFRP5 and different Xwnts. DIG-UTP labelled antisense probes against XsFRP5 (A–C), Xwnt2b (D–F), Xwnt5a (G–I) and Xwnt11R (J–L) were used for whole-mount *in situ* hybridisation of stage 35 embryos (A, D, G, J), stage 40 embryos (B. E, H, K) and stage 40 gut tube and heart-explants (C, F, I, L). Corresponding sections are shown in A′–K′. he = heart, li = liver, lu = lung, st = stomach/duodenum, oe = oesophagus, and pr = pronephros.

observed expression in the heart and a very restricted endodermal expression in the oesophagus (Fig. 5L).

To address the question if Xwnt2b, which is expressed in stage 35 *Xenopus* embryos in a pattern complementary to the one of XsFRP5, and which we have found to interact with and to be efficiently inhibited by XsFRP5, could be its *in vivo* target, we knocked down Xwnt2b signalling activity in the *Xenopus* embryo. For this purpose, three different antisense morpholino oligonucleotides were generated; a splice-morpholino directed at the exon3/intron3 transition sequence of the Xwnt2b mRNA (2b-SpliMO), a second splice-morpholino directed at the intron3/exon4 transition (2b-SpliMO2) and a third MO directed at the start site of translation (2b-MO) were designed (see Materials and methods for details). RT-PCR analysis of SpliMO injected embryos (Fig. 6A) reveals significantly reduced levels of fully spliced Xwnt2b mRNA and appearance of two additional, longer RNA products which contain sequence elements of intron3 joined to exon3 from the *Xwnt2b* gene (data not shown); the appearance of a smaller RT-PCR product could reflect SpliMO-induced skipping of exon3. In addition, the overall quantity of these different forms of Xwnt2b mRNA appears to be reduced, indicative of degradation of Xwnt2b mRNA that is not properly spliced. RNA preparations from 2b-SpliMO2 injected embryos equally reveal overall reduced levels of properly spliced Xwnt2b mRNA as well as the appearance of a longer, presumably incompletely spliced RNA variant (Supplementary fig. 2B). 2b-MO is a specific inhibitor of Xwnt2b translation *in vitro* (Supplementary fig. 2A).

Injection of these different MOs into the prospective endoderm and mesoderm (as revealed by β-Gal tracing), followed by analysis of marker gene expression of stage 40 embryos in pancreas, stomach, liver and intestine revealed that Xwnt2b knockdown results in a size reduction of the stomach (Figs. 6F, G, K and Supplementary figs. 2C–E), as well as in pancreatic hypoplasia, affecting both dorsal and ventral anlagen (Figs. 6D, E and Supplementary figs. 2F–H). Hepatic and intestinal expression of fibrinogen was unaffected (Figs. 6H, I and Supplementary figs. 2I–K). Size reduction of the stomach as a result of Xwnt2b knockdown could be explained by a positive function for Xwnt2b in proliferation control. In order to address this issue, the absolute number of proliferating cells was determined on sections prepared from embryos stained for pH3. It turns out that the total number of endodermal pH3 staining is indeed markedly reduced in stage 37/38 and 40, but not in stage 32 embryos upon injection of 2b-SpliMO (Fig. 6J). In line with these late effects on

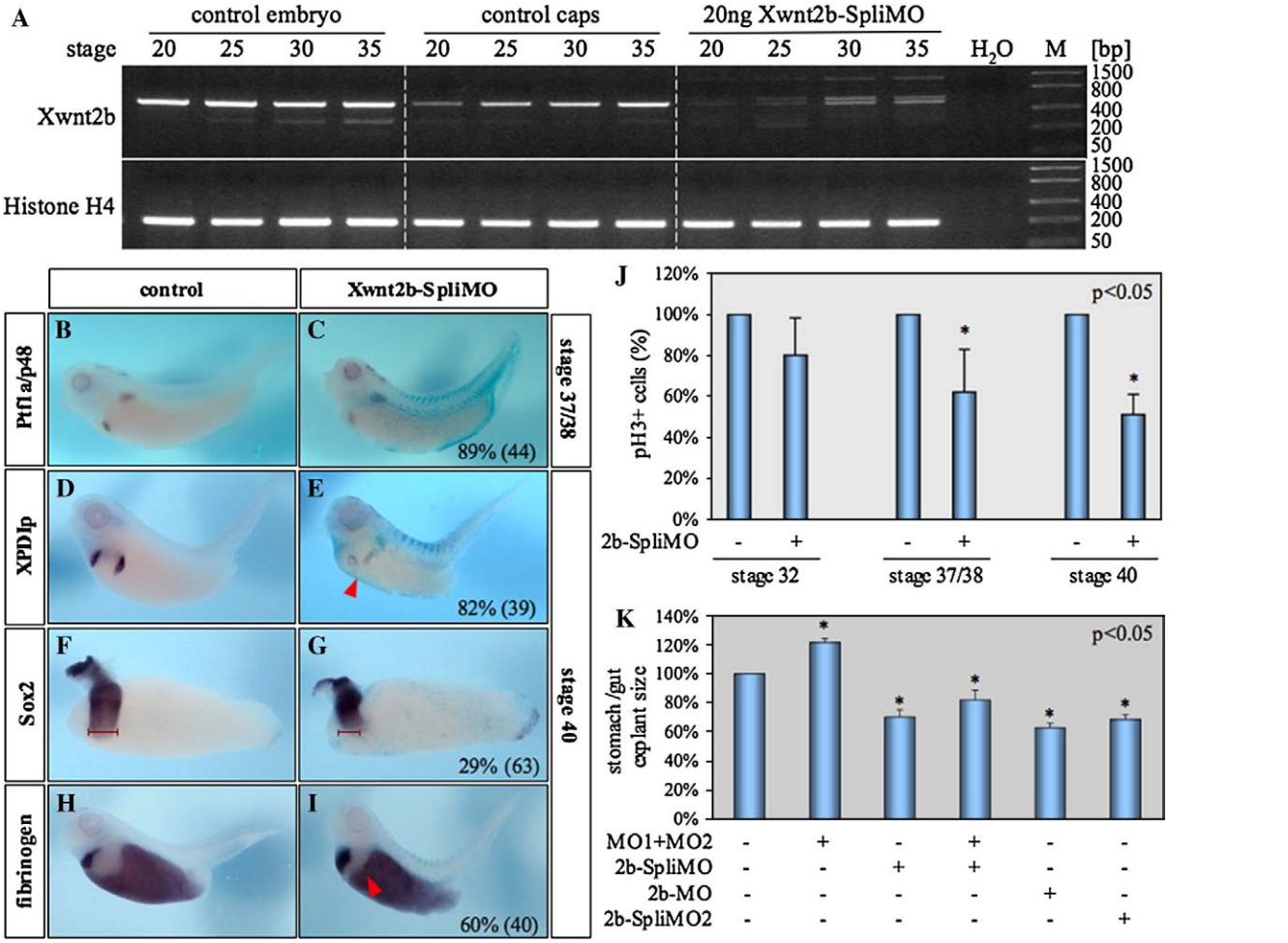

Fig. 6. Knockdown of Xwnt2b expression causes foregut hypoplasia. (A) Specificity control for the Xwnt2b-Splice Morpholino (SpliMO). 20 ng SpliMO were injected animally into both blastomeres at the two cell stage and embryos/animal cap explants were analysed for the Xwnt2b transcript by RT-PCR at different developmental stages. M = Fastruler™ DNA ladder Low Range (Fermentas). (B–I) Marker gene expression analysis after Xwnt2b knockdown by whole-mount *in situ* hybridisation. A total amount of 20 ng SpliMO was injected into the vegetal pole of all four blastomeres at the four-cell stage. LacZ activity was used as a lineage tracer. Whole-mount *in situ* hybridisation was performed with stage 37/38 (B, C) and stage 40 embryos (D–I). The red arrowheads mark the hypoplastic pancreas (E) and stomach/duodenum (I) as detected by staining with XPDlp and fibrinogen probes, respectively. The red bars indicate the width of the Sox2 expression domain (F, G). (J) Vibratome sections of control and SpliMO-injected embryos, as well as of gut explants were analysed for proliferative cells using pH3-labelling. 10 consecutive sections of 3 embryos/explants were scored for $pH3^+$ cells in the anterior endoderm at stages 32 and 37/38, and in the stomach/pancreas/liver area of stage 40 gut explants. The mean value of $pH3^+$ cells in the controls was set to 100%. Significance was calculated using Student's *T* Test and marked with asterisks. (K) Quantification of the size of the Sox2-expression domain in control, MO1 + MO2-, Xwnt2b-SpliMO- and double injected gut explants as well as Xwnt2b-MO and Xwnt2b-SpliMO2 injected gut explants. The average pixel number in the controls was set to 100%. Significance was calculated using Student's *T* Test and marked with asterisks.

proliferation, earlier (stage 37/38) expression of Ptf1a/p48 was not significantly affected in Xwnt2b morphant embryos (Figs. 6B, C). Thus, as compared to XsFRP5 knockdown, partial loss of Xwnt2b yielded a reciprocal phenotype in respect to stomach size and proliferation activity in the endoderm.

Discussion

We have provided direct experimental evidence that a member of the secreted Frizzled-related protein family, *Xenopus* sFRP5, can assemble into one complex with and inhibit the signalling activity of multiple Wnt ligands, thereby interfering with both, canonical and non-canonical signalling. Knockdown of XsFRP5 results in early, transient hypoplasia of the pancreas. Furthermore, reduced XsFRP5 activity in *Xenopus* embryos leads to stomach enlargement. Xwnt2b, which is inhibited by XsFRP5 in its ability to activate canonical Wnt signalling in the *Xenopus* embryo and co-immunoprecipitate with XsFRP5 from programmed embryonic extracts, is also expressed in a complementary pattern with XsFRP5 during tailbud stages in the stomach/liver/pancreas territory. Knockdown of Xwnt2b results in hypoplasia of stomach and pancreas in tadpole stages of development. We also found that XsFRP5 can substitute for RA in VegT-programmed animal cap explants in respect to the induction of exocrine pancreatic marker gene expression.

XsFRP5 acts as a multi-specific inhibitor of canonical and non-canonical Wnt signalling

While all of the canonical and non-canonical Wnts analysed in this study were inhibited at least to some extent by high doses of XsFRP5 in the axis duplication assay, not all of these signalling molecules were found to co-precipitate efficiently with XsFRP5; the axis inducing activity of Xwnt8b, for example, is already significantly inhibited at the lowest dose of XsFRP5 tested, while it is only very weakly detectable in a complex with XsFRP5 in the co-immunoprecipitation assay. This finding raises the possibility that XsFRP5 could make use of different modes of operation as Wnt antagonist. In addition to preventing Wnt binding to the cognate receptors by forming a common complex (with or without other proteins), it could also bind to and inhibit other proteins involved in Wnt mediated activation of signal transduction, such as the Wnt receptors themselves, as has been proposed earlier (Bafico et al., 1999), or known co-receptors, such as LRP5/6. The latter, indirect mechanism for Wnt inhibition has indeed been defined for the Wnt antagonist Dkk (Mao et al., 2001). Similarly, efficient co-immunoprecipitation is not predictive for efficient inhibition of secondary axis induction, as in the case of Xwnt3a, which is pulled down efficiently but only marginally inhibited; thus, in addition to binding the Wnt ligands themselves, XsFRP5 may interact with multiple components of the Wnt signal receiving and transducing machinery, and each one of these individual interactions could contribute to efficiency and specificity of Wnt inhibition. However, irrespective of the exact molecular mechanism of its function, XsFRP5 is an antagonist that works on multiple Wnt ligands, both canonical and non-canonical ones; a positive, synergistic activity on Wnt signalling was not detected for any of the Wnt ligands tested here. These properties make sFRP5 also an interesting candidate reagent in preventing tumor cell proliferation; epigenetic inactivation of sFRP5 was reported to be associated with unfavourable prognosis of human breast cancer (Veeck et al., 2008). It remains to be established, if other members of the sFRP protein family can function as multi-specific Wnt inhibitors with a broad range of specificity comparable to the one of XsFRP5 (for a recent review on the information available see Bovolenta et al., 2008).

A function for XsFRP5 in endodermal organ specification and/or growth?

Xenopus sFRP5 knockdown embryos exhibited inhibition of pancreas specification, but nevertheless returned to almost normal levels of Ptf1a/p48 and XlHbox8 staining during differentiation/proliferation stages. Interestingly, a biphasic mode of Wnt-mediated regulation has been postulated for pancreas development in the mouse; early β-catenin activation prevents proper formation of pancreatic precursor cells, whereas later in development, β-catenin activation promotes proliferation and thereby gross enlargement of the exocrine pancreas (Dessimoz et al., 2005; Heiser et al., 2006; Murtaugh et al., 2005). In analogy, according to the results reported in this communication, XsFRP5 could modulate a primary Wnt signalling event in the context of pancreatic specification control in *Xenopus* embryos during tailbud stages of development, when XsFRP5 expression overlaps with hepatic and ventral pancreatic precursor cells (Pilcher and Krieg, 2002; Fig. 7). Furthermore, we also observed an increased endodermal proliferation as a consequence of XsFRP5 knockdown; such increased and perhaps premature proliferation activity could thus possibly interfere with proper pancreas specification. However, at this point, we cannot exclude the possibility of cell death contributing to the observed pancreas hypoplasia.

Even though XsFRP5 is expressed in the foregut endoderm at the time and place of hepatic induction in frogs and mice (Finley et al., 2003; Pilcher and Krieg, 2002), we did not observe effects on fibrinogen expression in the liver and posterior endoderm of tadpole stage *Xenopus* embryos upon XsFRP5 knockdown. Similarly, sFRP5 knockout has not been found to affect Hex expression that marks hepatic precursor cells in the mouse (Leaf et al., 2006). These findings

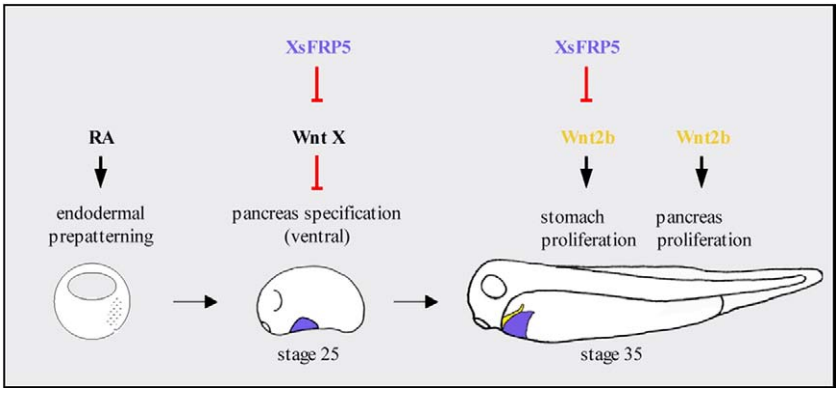

Fig. 7. A schematic drawing illustrating XsFRP5 function during endodermal organogenesis in *Xenopus*. RA signalling is needed to establish a prepattern in the endodermal cell population during gastrulation. During early somite stages XsFRP5 expression in the endoderm overlaps with the ventral pancreatic precursor cells and might inhibit the function of a putative WntX, thereby permitting pancreas specification. At tadpole stage, XsFRP5 expression is adjacent to mesodermal Xwnt2b expression, thereby modulating proliferative activities in the endoderm.

do not contradict a possible requirement for suppression of Wnt signalling in the anterior endoderm as being involved in hepatic development, since other, redundant Wnt-antagonists, such as Dkk1, Frzb1 and Crescent, could serve a similar function. Indeed, McLin et al. (2007) reported that ectopic activation of canonical Wnt signalling in the anterior endoderm by means of mRNA injection did interfere with liver development, perhaps by direct repression of Hex expression. Another possible explanation for the lack of an XsFRP5 knockdown effect on liver development in the frog is provided by the assumption that sFRPs act as modulators in the "fine-tuning" of Wnt signalling, with a knockdown resulting only in a moderate increase of endogenous Wnt activities. Conversely, ectopic activation of Wnt signalling as performed by McLin et al. (2007) is likely to result in a robust increase of Wnt activity that then interferes with liver development; liver specification was disturbed at such relatively high levels of Wnt signalling only when induced at early phases (between gastrula and early somite stages) of development. Finally, since the knockdown effect that we have obtained for XsFRP5 may only be partial with some degree of XsFRP5 activity remaining in the manipulated embryos, we cannot exclude the possibility of a concentration-dependent function; in such a scenario, full inhibition of XsFRP5 might be required to generate a liver phenotype. Interestingly, after this study was initially submitted for publication, a closely related analysis of XsFRP5 function in the context of foregut specification in *Xenopus* was published and liver hypoplasia as a result of XsFRP5 knockdown was indeed observed (Li et al., 2008); due to different injection protocols, a higher effective MO dose was achieved, providing an explanation for this apparent contradiction in respect to the results obtained in their and our study.

A function for Wnt signalling in the choice between endocrine and exocrine pancreatic fate?

We have previously demonstrated that RA signalling is essentially required for normal pancreas development in the frog, but also for the induction of endo- and exocrine pancreatic marker gene expression in VegT-programmed animal cap explants (Chen et al., 2004; Pan et al., 2007); in the absence of RA, VegT is sufficient to induce the liver-specific gene programme, including the liver precursor cell marker Hex and the differentiation marker Ttr. VegT injected animal cap explants express several different Wnt ligands (data not shown); while coinjection of VegT and XsFRP5 did not inhibit or increase Ttr expression as a marker gene for liver differentiation, XsFRP5 was found to substitute for the need of RA in respect to the induction of exocrine-, but not endocrine-specific gene expression. In line with these observations, we have previously observed that β-catenin enhances insulin expression in animal cap explants (Pan et al., 2007) and McLin et al. (2007) reported that repression of β-catenin signalling in the posterior endoderm results in ectopic exocrine, but not endocrine pancreas development. The exact molecular mechanism that is reflected in the functional link of RA signalling and inhibition of Wnt signalling for the formation of exocrine pancreatic tissue as reported in this communication remains to be established, but these findings could be of interest for the attempts in other cell systems to generate pancreatic cells from multipotent precursor cells.

Xenopus Wnt2b: a target for XsFRP5 in the endoderm?

Both, functional assays and expression characteristics define Xwnt2b as a primary candidate target protein for XsFRP5 in organogenesis stage *Xenopus* embryos. Morpholino antisense oligonucleotide mediated knockdown of Xwnt2b does indeed result in a phenotype complementary to the one observed for XsFRP5 knockdown, i.e. reduced rather than increased size of the developing stomach at embryonic stage 40. One possible interpretation for this effect is that Xwnt2b signalling could positively regulate the growth of this organ, and that this activity is negatively modulated by XsFRP5. Mesenchymal expression of two other Wnt antagonists, sFRP1 and sFRP2, was previously proposed to permit stomach epithelial differentiation in the mouse (Kim et al., 2005), perhaps reflecting inhibition of other Wnts in the context of later differentiation events in this organ.

Similar to the effects observed upon knockdown of XsFRP5, MO-mediated inhibition of Xwnt2b expression is also found to result in pancreas hypoplasia. This effect could reflect a positive regulatory function for Xwnt2b in the proliferation of the pancreas during later stages of gut development, when XsFRP5 and Xwnt2b are expressed in distinct territories. In line with such a scenario, we have found that XsFRP5 exerts an early, negative regulatory role in proliferation, while Xwnt2b has an opposite, stimulatory effect on endodermal proliferation in later stages of organogenesis. Interestingly, ectopic expression of Wnt1 and Wnt5a under the control of the Pdx1 promoter in the mouse is associated with stomach–duodenal re-patterning, pancreas and spleen agenesis, as well as with a reduced size of pancreas, spleen and stomach (Heller et al., 2002). This correlates with data obtained in *Xenopus*, showing that early misexpression of canonical Wnt signalling is inhibitory to foregut development (McLin et al., 2007), and it is also in line with our own results, showing that the loss of the Wnt antagonist XsFRP5 results in increased cell proliferation at stage 32 and a reduced ventral pancreatic anlage, most likely by de-repression of early acting, endogenous Wnt signals. Non-neural expression of Xwnt2b is first detectable at stage 30 (Landesman and Sokol, 1997) and the knockdown of Xwnt2b did not result in a significant decrease in cell proliferation before stage 37/38. As mentioned above, a biphasic model for β-catenin signalling has been described for murine pancreas development, with pancreatic hyperplasia when activated at later stages (Heiser et al., 2006). In analogy, we propose that Xwnt2b mediates its proliferative cues via canonical Wnt signalling during later phases of endoderm patterning. Consistently, we found that Xwnt2b morphant embryos expressed normal levels of Ptf1a/p48, pointing towards unaffected pancreatic specification, whereas the expression of the exocrine differentiation marker XPDIp was grossly reduced, perhaps resulting from decreased cell proliferation.

In conclusion, data reported in this study lead us to propose that, during early embryonic development, XsFRP5 could exert a positive function in the formation of pancreatic precursor cells by down-regulating proliferation-promoting and specification-inhibiting Wnt signal(s), which remain to be identified. Li et al. (2008), in good agreement with our own observations, reported that knockdown of XsFRP5 results in hypoplastic ventral pancreatic buds; they further demonstrate that increased Wnt11 signalling is responsible for this effect. Thus, Wnt11 constitutes a prime candidate for the early acting Wnt signal that needs to be repressed by XsFRP5 in order to allow for proper pancreas specification. During later stages of development, proliferation of stomach and pancreas could be positively regulated by Wnt signalling. In this phase of gut development, Xwnt2b is expressed in the mesoderm adjacent to the pancreas/stomach forming endoderm (Fig. 7).

Acknowledgments

We are grateful to J. Christian, P. Krieg, M. Kühl, E. Pera and S. Sokol for generously providing constructs used in this study and thank M. Dornwell for technical assistance and A. Borchers for critical reading of the manuscript. This work was supported by funds from the Deutsche Forschungsgemeinschaft to T.P.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.004.

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