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Different thresholds of Wnt-Frizzled 7 signaling coordinate proliferation, morphogenesis and fate of endoderm progenitor cells

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Summary

Wnt signaling has multiple dynamic roles during development of the gastrointestinal and respiratory systems. Differential Wnt signaling is thought to be a critical step in *Xenopus* endoderm patterning such that during late gastrula and early somite stages of embryogenesis, Wnt activity must be suppressed in the anterior to allow the specification of foregut progenitors. However, the foregut endoderm also expresses the Wnt-receptor Frizzled 7 (Fzd7) as well as several Wnt ligands suggesting that the current model may be too simple. In this study, we show that Fzd7 is required to transduce a low level of Wnt signaling that is essential to maintain foregut progenitors. Foregut-specific Fzd7-depletion from the *Xenopus* foregut resulted in liver and pancreas agenesis. Fzd7-depleted embryos failed to maintain the foregut progenitor marker *hhex* and exhibited decreased proliferation; in addition the foregut cells were enlarged with a randomized orientation. We show that in the foregut Fzd7 signals via both the Wnt/β-catenin and Wnt/JNK pathways and that different thresholds of Wnt-Fzd7 activity coordinate progenitor cell fate, proliferation and morphogenesis.

Keywords

Frizzled 7; Wnt; β-catenin; JNK; endoderm; foregut patterning; *hhex*; *vent2*; *Xenopus*

Introduction

The epithelial lining of the digestive and respiratory systems and organs such as liver, pancreas, and lungs are derived from the embryonic endoderm. The endoderm germ layer is specified during gastrulation and is then patterned along the anterior-posterior (A-P) axis into broad foregut and hindgut progenitor domains, which become progressively subdivided into specific organ lineages by a reiterative series of Wnt, FGF and BMP growth factor signaling events (Zaret, 2008; Zorn and Wells, 2009). These pathways are highly dynamic

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and in just a few hours of embryogenesis, or at different ligand concentrations, the same signals can have dramatically different effects on the same population of endoderm cells (McLin et al., 2007; Serls et al., 2005; Wandzioch and Zaret, 2009). The molecular mechanisms that regulate the spatial-temporal activity of these pathways during endoderm organogenesis are poorly understood. A detailed knowledge of these complex signaling events will facilitate efforts to direct the differentiation of human stem cells into different endoderm lineages (Kroon et al., 2008; Si-Tayeb et al., 2010; Spence et al., 2011; Zaret, 2008).

Wnt signaling is particularly dynamic during endoderm organogenesis. In *Xenopus* and zebrafish, maternal Wnt/β-catenin signaling initially promotes gastrulation and anterior endoderm fate during germ layer formation (Rankin et al., 2011; Schier and Talbot, 2005; Zorn et al., 1999; Zorn and Wells, 2007). Only hours later between mid-gastrula and early somite stages zygotic Wnt signals have the opposite affect and repress foregut fate in the anterior endoderm while promoting hindgut fate in the posterior endoderm (Goessling et al., 2008; McLin et al., 2007). After patterning into foregut and hindgut progenitors domains, distinct Wnt signals then promote the specification, differentiation and/or outgrowth of the lungs, liver, pancreas, stomach and intestine (Lade and Monga, 2011; Murtaugh, 2008; Poulain and Ober, 2011; Shin et al., 2011; Verzi and Shivdasani, 2008).

Our previous studies on the role of Wnt-signaling in *Xenopus* endoderm patterning suggest that multiple Wnt ligands from the lateral plate mesoderm including Wnt5a, 5b, 8 and 11 signal via both the canonical Wnt/β-catenin and the non-canonical Wnt/JNK pathways to promote hindgut fate and morphogenesis in the posterior endoderm (Li et al., 2008; McLin et al., 2007). In the canonical pathway binding of Wnt ligands (such as Wnt8 and Wnt11) to Frizzled and LRP5/6 receptors causes the accumulation of nuclear β-catenin, which interacts with TCF/LEF transcription factors (Clevers, 2006; MacDonald et al., 2009) to activate target genes that promote posterior endoderm fate including the homeobox genes *vent1* and *vent2* (collectively referred to here as *vent1/2*) (McLin et al., 2007). There is evidence suggesting that Wnt11 and/or Wnt5a/b also activate a β-catenin-independent Wnt/JNK pathway in the endoderm, which signals via Rho-family GTPases and Jun-N-terminal-kinase (JNK) (Kim and Han, 2005; Wallingford and Habas, 2005) to regulate cytoskeleton dynamics, cell polarity and cell shape changes during gut morphogenesis (Li et al., 2008; Reed et al., 2009), although the precise cellular mechanisms are poorly understood.

In the anterior endoderm the Wnt-antagonist Sfrp5 suppresses both the Wnt/β-catenin and Wnt/JNK pathways to promote foregut development (Li et al., 2008). This has led to the model where “Wnt-OFF” promotes foregut progenitors and “Wnt-ON” specifies hindgut progenitors. However, this model may be too simplistic. Sfrps have recently been shown to exhibit biphasic activity: repressing Wnts at high concentrations but facilitating Wnt ligand diffusion and signaling at low concentrations (Mii and Taira, 2009). Moreover both Wnt11 and its putative receptor Frizzled 7 (Fzd7) are expressed in the foregut endoderm (Djiane et al., 2000; Li et al., 2008; Medina et al., 2000; Wheeler and Hoppler, 1999). These observations led us to hypothesize that Fzd7 may mediate a low level of Wnt signaling important for foregut progenitor development.

Although the role of *fzd7* in the foregut endoderm is unknown, its function in *Xenopus* axis specification and gastrulation has been well studied. In this context, gain-of-function and in vitro studies have shown that Fzd7 can interact with various Wnt ligands, (including Wnt5a, 8b and 11) and activate either canonical or non-canonical Wnt pathways (Brown et al., 2000; Djiane et al., 2000; Medina et al., 2000; Medina and Steinbeisser, 2000; Sumanas and Ekker, 2001). Loss-of-function studies indicate that maternal Fzd7 signals via the Wnt/β-catenin pathway in dorsal axis specification (Sumanas and Ekker, 2001; Sumanas et al., 2000), whereas zygotic Fzd7 in the chordomesoderm regulates gastrulation cell movements of via several non-canonical Wnt pathways. Specifically, Fzd7 activation of a PKC pathway regulates tissue separation of the mesoderm and ectoderm, whilst Fzd7/JNK regulates convergent extension of the axial mesoderm (Kim et al., 2008; Medina et al., 2004; Sumanas and Ekker, 2001; Winklbauer et al., 2001).

In this study we used targeted microinjection of fzd7 morpholinos (fzd7-MO) to specifically deplete Fzd7 from the foregut endoderm. We demonstrate that Fzd7 is required to mediate a low level of both Wnt/β-catenin and Wnt/JNK signaling that coordinates foregut progenitor fate, proliferation and morphogenesis. Both Fzd7/β-catenin and Fzd7/JNK pathways contributed to foregut fate and proliferation, whereas the JNK pathway (but not β-catenin signaling) regulated cell morphology. Our data support a revised model of endoderm patterning where Wnt signaling has different thresholds along the A-P axis such that high Wnt activity promotes hindgut over foregut fate, but that a low essential threshold of Wnt-Fzd7 activity is required to maintain foregut progenitors.

Material and Methods

Embryo manipulations and microinjections

Embryo manipulation and microinjections were performed as described previously (McLin et al., 2007). To specifically target the foregut endoderm and avoid the chordomedoserm we injected fzd7-MOs and the various mRNAs used in this study (along with a lineage tracer to confirm targeting) into the D1 cells of 32-cell stage embryos, which give rise to the foregut (Moody, 1987). To knockdown both *Xenopus laevis* Fzd7 homeologs we injected a mixture of two characterized translation-inhibiting fzd7-MOs (25 ng each) (Sumanas and Ekker, 2001): 5-CCGGCTCCAACAAGTGATCTCTGG-3 and 5-GCGGAGTGAGCAGAAATCGGCTGAT-3. The following mRNAs were used: pCS107-Fzd7, pT7TS-Sfrp5, pCS107-Dkk1 (Li et al., 2008), and GR-Lef-βCTA (Domingos et al., 2001). The following plasmids were used: pCS2+c.a.JNK (Liao et al., 2006). Dexamethasone (1 μM; for GR constructs) and the following cell-soluble inhibitors were dissolved in DMSO and added to the media at stage 11; JNK inhibitor SP600125 (50–100 μM), Rac1 inhibitor NSC23766 (100–200 μM), Cdc42 inhibitor Casin (50 μM), PKC inhibitor BIM (40 μM), Ca2+-dependant PKC inhibitor Go6976 (40 μM), and CamKII inhibitor, KN-93 (20 μM), Axin inhibitor XAV-939 (10–80 μM). Inhibition of cell proliferation was achieved by addition of hydroxyurea (HU, 20 mM) to media at stage 9 and incubated until stages 12 and 19, as previously described (Ohnuma et al., 1999).

In situ hybridization and immunohistochemistry

In situ hybridization and immunohistochemistry were performed as previously described (McLin et al., 2007; Sinner et al., 2004). The following primary antibodies were used: rabbit anti-β-catenin (1:250; H-102, Santa Cruz Biotechnologies), mouse anti-C-cadherin (1:200; 6B6, DSHB), mouse anti-E-cadherin (1:200; 5D3, DSHB), mouse anti-β1-integrin (1:500; 8C8, DSHB), rabbit anti-atypical-PKC (1:100; sc-216 Santa Cruz Biotechnologies), rabbit anti-phospho-histone H3 (1:250; Cell signaling), rabbit anti-Fzd7 (1:200; R&D systems), rabbit anti-active-caspase-3 (1:250; BD Pharmigen). The following secondary antibodies were used: goat anti-rabbit-cy5, goat anti-rabbit-cy2 or goat anti-mouse-cy5 (1:300; Jackson Immunoresearch). Nuclei were counterstained with Topro-3. In all experiments exactly the same confocal and camera settings were used for control and manipulated sibling embryos.

TOP:Flash and AP1:Luciferase assay

Top-flash (150 pg), AP1:luciferase (150 pg; Stratagene), and pRL-TK renilla (25 pg) (Li et al., 2008) plasmids were injected into embryos as indicated in the text. Each experiment was performed in triplicate using five embryos per replicate, and luciferase activity was measured using a commercial kit (Promega). Luciferase activity was normalized to co-injected TK-renilla and the mean relative activity of the triplicate samples was shown +/− S.D. Each experiment was repeated a minimum of 3 times and a representative result is shown.

Western blot

Western blots were carried out as described (Cha et al., 2008). Antibodies concentrations were: rabbit anti-pJNK, (1:750; Cell Signaling); rabbit anti-total JNK, (1:750; Cell Signaling); mouse anti-C-cadherin (1:500; DSHB), mouse anti-E-cadherin (1:500; DSHB); and mouse anti-tubulin (1:5000; Neomarker).

Results

Graded reduction in Wnt signaling differentially impacts endoderm progenitor fate

The current model of endoderm patterning in *Xenopus* predicts that “Wnt-ON” promotes hindgut fate in the posterior, whereas “Wnt-OFF”, due to the Wnt-antagonist Sfrp5, promotes foregut fate (Li et al., 2008; McLin et al., 2007). Although the posterior expression of *wnt8*, *wnt5a* and *wnt5b* mRNAs are consistent with this model (Li et al., 2008; McLin et al., 2007) close examination of *wnt11* and its putative receptor *fzd7* indicate that they are expressed in the foregut endoderm underlying the *sfrp5* expression domain at stage 19 (Li et al., 2008; Supplementary Fig. S1). This suggests that the current model may be too simplistic and led us to hypothesize that a low level of Wnt-Fzd7 signaling might have a positive role in foregut progenitor development.

To test the hypothesis that a low level of Wnt signaling is required for foregut development, we microinjected an increasing doses of mRNA encoding Sfrp5 into the anterior endoderm and assayed the expression of the foregut marker *hhex* and hindgut markers *vent1/2*. A moderate dose of *sfrp5* (500–800 pg mRNA) expanded the *hhex* expression at the expense of vent1/2-expressing hindgut domain (Fig. 1E–G, J–L), which is consistent with our

previous findings (Li et al., 2008). However, at higher doses of *sfrp5* (2–3 ng), rather than expanded *hhex* we observed a loss of *hhex* expression as well as reduced *vent1/2* expression (Fig. 1I,N). The non-cell autonomous effects on the hindgut endoderm were expected as secreted Sfrp5 is predicted to readily diffuse (Mii and Taira, 2011).

Since Sfrps can sometimes (at low concentrations) potentiate Wnt signaling we confirmed the Sfrp5 results by inhibiting Wnt signaling using an alternative method: We treated embryos from stages 11–19 with a does range of the cell-soluble small molecule Wnt-inhibitor XAV-939; a tankyrase-inhibitor that stabilizes Axin and thus promotes degradation of cytosolic β-catenin (Huang et al., 2009). Recapitulating the dose-dependent effects of Sfrp5, low concentration of XVA-939 (modest Wnt inhibition) expanded *hhex*, whereas high concentrations of XVA-939 repressed both *hhex* and *vent1/2* (Supplementary Fig. S2).

These results support the hypothesis that a low level of Wnt signaling is actually required for foregut development, with hindgut progenitors requiring an even higher level of Wnt activity.

Fzd7 is required for foregut organogenesis

We next wanted to use a loss-of-function approach to test the role of Wnt signaling in the foregut. Since multiple secreted Wnt ligands are expressed in the ventral region of the embryo at this time in development we focused on the role of the Wnt receptor Fzd7. In addition to being expressed in the foregut endoderm, *fzd7* is also strongly expressed in the axial mesoderm (Supplementary Fig. S1), and previous global knockdown approaches examining its role in gastrulation (Djiane et al., 2000; Medina et al., 2000; Sumanas and Ekker, 2001; Winklbauer et al., 2001) precluded analysis of later digestive system development. To test the function of Fzd7 specifically in the foregut without disturbing its mesodermal role in gastrulation, we injected a mixture of two well-characterized translation-blocking Fzd7 antisense morpholino oligos (fzd7-MOs) (Sumanas and Ekker, 2001) together with a red fluorescent tracer into D1 cells of 32-cell stage embryos, which are fated to give rise to the ventral foregut endoderm (Moody, 1987). Lineage analysis confirmed that the fzd7-MOs were limited to the foregut (Fig. 2A). Moreover these foregut-targeted embryos did not exhibit defects in either convergent extension or mesoderm-ectoderm tissue separation, whereas control injections targeting the dorsal mesoderm recapitulated the published gastrulation defects, confirming the efficacy of the fzd7-MOs (Supplementary Fig. S3).

Depletion of Fzd7 protein from the membrane of foregut endoderm cells was confirmed by immunostaining at stage 19 (Fig. 2B,C). When cultured until organ bud stages (42–45) approximately 75% (n=35) of the fzd7-MO embryos exhibited dramatic gut defects (Fig. 2N,O). Histology and examination of isolated gut tubes revealed disrupted gut coiling, foregut edema, and severe organ hypoplasia with little if any heart, liver or pancreas tissue and a reduced stomach in most Fzd7 morphants (Fig. 2P–S). To determine whether endoderm patterning and organ specification was compromised, we examined various foregut markers (Fig. 2) at multiple developmental stages. Initial expression of *hhex* in the gastrula anterior endoderm was unaffected (data not show), but by stage 19 *hhex* expression was dramatically down regulated in the foregut progenitors of Fzd7-depleted embryos (Fig.

2E,F), whereas expression of the pan-endodermal marker *sox17* was not changed (data not shown). At stage 35, when organ lineages are specified, Fzd7 morphants failed to express liver (*nr1h5*; previously *for1*, Xenbase.org) and pancreas (*pdx1*) markers (Fig. 2H,I,K,L). Expression of the cardiac differentiation marker *tnni3* was not significantly altered (data not shown), suggesting that the heart defect in Fzd7 morphants at stage 42 was due to impaired cardiac morphogenesis and not a failure of heart specification.

Analysis of *alpha-2-macroglobulin* (*a2m*; previously *edd*, Xenbase.org) expression, which is expressed in both the liver and the presumptive intestine, suggested that hindgut fate was not compromised (Fig. 2R,S). The fact that *a2m* was not ectopically expressed in the remnant foregut tissue of Fzd7 morphants argues that the foregut progenitors did not adopt a hindgut fate as is the case when Wnt/β-catenin is hyper-activated in the post-gastrula anterior endoderm (McLin et al., 2007).

To confirm that the Fzd7 morphant phenotype was specifically due to loss of Fzd7, we co-injected the fzd7-MOs along with a synthetic *fzd7* mRNA lacking MO-target sequence, which was sufficient to rescue Fzd7 immunostaining in foregut cells and restore foregut gene expression (Fig. 2D, G, J and M). We conclude that Fzd7 is required to maintain foregut progenitors and for subsequent foregut organogenesis.

Fzd7 is required for foregut cell morphology

We noticed from the residual Fzd7 immunostaining that Fzd7-depleted foregut cells had abnormal morphology. Since Fzd7 can activate non-canonical Wnt signaling to regulate cytoskeleton dynamics and cell adhesion in other contexts (Djiane et al., 2000; Medina et al., 2000), we examined this more carefully. Removing the neural plate to observe the surface of the foregut endoderm at stage 19, we found that the Fzd7-depleted foregut cells were enlarged and loosely adherent in comparison to controls (Fig. 3A,B). Cell adhesion and cell shape are regulated by interactions between cell surface adhesion molecules such as Cadherins, which in turn are linked to the actin cytoskeleton by Catenins (Adams et al., 1996; Tao et al., 2007). Immunostaining showed that while control foregut cells were arranged in an organized polygonal array, Fzd7-depleted cells were larger, round and disorganized, typical of reduced cell adhesion (Rozario et al., 2009; Witzel et al., 2006). Many of the enlarged fzd7-MO foregut cells exhibited reduced C-cadherin and β1-integrin at cell membrane as well as reduced levels of cortical β-catenin and F-actin at the inner cell surface (Fig. 3C–J). This effect was more mosaic for β-catenin and C-cadherin and correlated with cells that received a high dose of the fzd7-MO (based on co-injected lineage label; data not shown). Western blotting analysis of dissected stage 19 foreguts demonstrated that the total amount of C-cadherin and E-cadherin were not significantly changed (Fig. 3N), suggesting that the loss of Fzd7 impacts cadherin localization rather than expression.

To quantify the changes in cell size and polarity we measured the length, width and orientation of foregut cells in control and Fzd7-depleted embryos. In mid-sagittal sections of control embryos the long axis of foregut cells was predominantly vertical; oriented parallel to the dorsal-ventral axis. In contrast, the Fzd7-depleted foregut cells were significantly larger (although their length-to-width ratio was unchanged) and the orientation of their long

axes was randomized (Fig. 3 K–M). We also assayed spindle orientation in foregut cells undergoing mitosis by β-tubulin immunostaining. The mitotic spindles were similarly oriented along the long axis in controls cells but randomized in Fzd7 morphants (data not shown). Together, these data show that Fzd7 is required for foregut cell adhesion, size and orientation.

Fzd7 is required for foregut cell proliferation

During analyses of the mitotic spindles we observed fewer dividing cells in the foregut of Fzd7 morphants. Given the well-known role of Wnt signaling in regulating proliferation of multiple cells types we examined this in more detail. Immunostaining of phospho-histone H3 (PH3) to mark cells undergoing mitosis revealed that Fzd7-depleted embryos indeed had significantly fewer proliferating cells in the foregut at stage 19 (Fig. 4). Analysis of earlier stage embryos indicated that the reduced proliferation was evident as early as mid-gastrula (Fig. 4), prior to when we first observed defects in gene expression or cell morphology.

To test whether other defects in Fzd7 morphants were primarily due to the loss of proliferation, we treated blastula embryos with hydroxyurea (HU), which inhibits cell proliferation (Ohnuma et al., 1999). PH3 staining confirmed that HU treatment from the blastula stage reduced proliferation at stages 12 and 19 comparable to that of fzd7-MO-injected embryos (Supplementary Fig. S4). However the HU treated embryos did not exhibit any disruption in foregut cell morphology nor was there a loss of foregut gene expression. On the contrary HU treatment resulted in expanded *hhex* in the liver at stage 35 (Supplementary Fig. S4). This suggests that reduced proliferation alone cannot account for the loss of foregut identify in Fzd7 morphants. However, we postulate that the decreased proliferation may contribute to later foregut organ bud hypoplasia. TUNEL assays and active caspase-3 staining indicated that there was no significant cell death in either controls or Fzd7-depleted embryo at stage 19 (Supplementary Fig. S5).

We conclude that Fzd7 has multiple roles in the foregut including maintenance of cell proliferation, foregut gene expression and proper cell morphology. Moreover the data suggest that the disrupted gene expression and altered cell morphology in Fzd7 morphants is unlikely to be due primarily to reduced cell proliferation.

Fzd7 depletion results in reduced Wnt/β-catenin and Wnt/JNK activity

Fzd7 has been shown to stimulate canonical β-catenin, as well as non-canonical Wnt pathways in different contexts (Medina et al., 2000), although in most instances the activation of these different downstream pathways is thought to be mutually exclusive with the canonical and non-canonical pathways antagonizing one another (Nemeth et al., 2007; Topol et al., 2003). To better understand the molecular basis of Fzd7 function in the foregut we assayed the status of the Wnt/β-catenin and Wnt/JNK intracellular signaling pathways both of which are known to be active in the *Xenopus* endoderm at this time (Li et al., 2008).

To measure endogenous β-catenin/Tcf transcriptional activity downstream of canonical Wnt signaling we used a TOP:flash reporter plasmid, which contains multiple TCF DNA-binding sites driving luciferase expression. We injected the TOP:flash reporter, with or without the

fzd7-MOs, into D1 cells or D4 cells at 32-cell stage, which will develop into future foregut and hindgut, respectively (Moody, 1987) and measured luciferase activity at stage 19. As expected, the hindgut had higher endogenous β-catenin/Tcf activity than foregut. However control embryos did exhibit a modest level of reporter activity in the foregut, which was significantly reduced by fzd7-MO injection (Fig. 5A). We also examined levels of the activated C-terminal dephosphorylated form of β-catenin by western blot of foregut explants (Fig. 5B) and by measuring intensity of nuclear β-catenin immunostaining (Fig. 5C–E), both of which were detected at a low level in the foregut and dramatically reduced by Fzd7 depletion.

To measure non-canonical Wnt/JNK activity, we used an AP1:luciferase reporter plasmid (Cheyette et al., 2002), which contains AP1 (c-Jun/c-Fos) DNA-binding sites driving expression of Luciferase. Activated JNK phosphorylates c-Jun and stimulates c-Jun/c-Fos mediated transcription. Injecting the AP1:luciferase reporter into either the presumptive hindgut and foregut revealed that JNK was active in both regions, although slightly higher in the hindgut. Moreover Fzd7 depletion from the foregut resulted in significantly reduced AP1:luciferase activity (Fig. 5A), and western blot analysis of foregut explants confirmed that phospho-JNK levels were reduced in Fzd7 morphants (Fig. 5B).

These data demonstrate that in the foregut Fzd7 transduces a low but detectable level of both Wnt/β-catenin and Wnt/JNK signaling.

Fzd/β-catenin and Fzd/JNK coordinate foregut progenitor proliferation, gene expression and morphology

To determine whether different aspects of the Fzd7 morphant phenotype were due to reduced β-catenin and/or JNK signaling, we performed a series of loss of function and rescue experiments. First we specifically inhibited either the Wnt/β-catenin pathway (by microinjecting RNA encoding the canonical Wnt-antagonist Dkk1 in the anterior endoderm at 32-cell stage) or inhibited the Wnt/JNK pathway (by adding the JNK-inhibitor SB600125; 100 μM to the culturing medium at stage 11) and determined to what extent either of these could recapitulate the fzd7-MO phenotype. TOP:flash and AP1:luciferase assays confirmed that at stage 19 Dkk1 only inhibit β-catenin activity and did not impact JNK activity, whereas the JNK-inhibitor only repressed the AP1:luciferase and did not change TOP:flash activity (data not shown). PH3 immunostaining revealed that JNK inhibition caused a significant reduction in foregut cell proliferation at both stages 12 and 19, similar to Fzd7 morphants, whereas Dkk1 overexpression repressed foregut cell proliferation predominantly at stage 12 (Fig. 6A). This indicates that both β-catenin and JNK activity are required for foregut cell proliferation.

Next we examined foregut gene expression and found that the JNK-inhibitor or high levels of Dkk1 mRNA (1500 pg) could both suppress, but not totally eliminate, *hhex* expression (Fig. 6I, M, N). Lower doses of Dkk1 (< 500 pg) expanded *hhex* (data not shown) similar to what we observed with Sfrp5 low dose over expression (Fig. 1), which is consistent with the model that reducing, but not completely eliminating β-catenin activity, expands the foregut. Interestingly, different doses of JNK inhibition did not exhibit a similar bimodal impact on *hhex* expression and we never observed increased *hhex* expression at any dose of the JNK

inhibitor (data not shown). These data suggest that both β-catenin and JNK activity are required for robust foregut gene expression, and that β-catenin regulates foregut versus hindgut fate in a dose responsive manner.

We next examined cell morphology in the Dkk1-injected and JNK-inhibited embryos by immunostaining of cytoskeletal β-catenin. Dkk1 had no impact on cytoskeletal β-catenin even though it caused a reduction of the nuclear β-catenin, confirming the suppression of canonical Wnt signaling. In contrast, the JNK inhibitor caused enlarged foregut cells with reduced cortical β-catenin similar to Fzd7 loss of function (Fig. 6C,D,G,H). Prolonged JNK inhibition also prevented elongation of the endoderm that normally occurs between stages 15–30 (Supplementary Fig. S6). This observation is similar to previous reports of Sfrp5 and dominant negative Dsh overexpression (Li et al., 2008) consistent with a role for Wnt/JNK-mediated gut morphogenesis.

We also tested a number of other inhibitors to different intracellular effectors of non-canonical Wnt signaling including inhibitors of: CamKII, receptor coupled G-proteins, PI3 kinase, Cdc42, Rac1 and PKC. None of these had an obvious impact on foregut cell proliferation (data not shown). Rac1 inhibition partially phenocopied fzd7-MO by suppressing *hhex* expression, whereas Cdc42 and PKC inhibition caused an increase in the size of foregut cells, similar to the Fzd7 morphants (Supplementary Fig. S7; data not shown). These findings suggest Rac1, Cdc42 and PKC may also participate in non-canonical Wnt/Fzd7 signaling to regulate gene expression and/or cell morphology in the foregut.

To further confirm that the Fzd7 morphant phenotype was due to loss of both the Wnt/β-catenin and Wnt/JNK pathways we preformed rescue experiments co-injecting the fzd7-MOs with RNA encoding either constitutively active JNK (caJNK) (Liao et al., 2006) or a hormone inducible Lef1-β-catenin fusion construct (GR:Lef-βCTA, which constitutively activates Tcf/Lef-β-catenin targets in the presence of dexamethasone) (Domingos et al., 2001). We targeted these injections to the presumptive foregut endoderm, which avoids the axial mesoderm and as expected all the injected embryos gastrulated normally. Both caJNK (200 pg) and GR:Lef-βCTA (200 pg, induced at stage 11) partially rescued foregut proliferation (Fig. 6B) and *hhex* expression (Fig. 6K,L) in fzd7-MOs, whereas only the caJNK rescued cell morphology (Fig. 6E,F). Reporter assays demonstrated that caJNK only activated the AP1:luc reporter and that GR:Lef-βCTA only activated the TOP:flash reporter (data not shown).

In these rescue experiments we again observed a dose responsive effect in the Wnt/β-cat pathway. The same dose of GR:Lef-βCTA (200 pg) that rescued *hhex* in Fzd7-depleted embryos had the opposite effect and repressed *hhex* when injected into control embryos (89% n=19; not shown). This is probably because controls have endogenous Wnt/Fzd7 signaling and the injection elevates β-catenin activity above the threshold for foregut identity. Consistent with this, injection of a 3-fold higher dose of GR:Lef-βCTA RNA (600 pg) into fzd7-MO embryos no longer rescued *hhex* (90%, n=20, not shown). Unlike GR:Lef-βCTA, caJNK did not have a bimodal impact on gene expression and it never repress *hhex* at any of the doses tested. However, we did observe a caJNK dose effect on cell morphology with 200 pg of caJNK RNA rescuing the large cell size in the fzd7-MO as described above,

whereas 600 pg of caJNK resulted in smaller than normal, disorganized foregut cells (data not shown) similar to previous reports of elevated Wnt/JNK activity caused by Sfrp5-depletion (Li et al., 2008).

We conclude from these experiments that Fzd7 signals via both the β-catenin and JNK pathways in the foregut. Foregut progenitor proliferation and gene expression require both Fzd7/β-catenin and Fzd7/JNK signaling, whereas the JNK, but not the β-catenin, pathway regulates foregut cell morphology. Moreover the data are consistent with the hypothesis that a low level of Wnt/Fzd7 activity promotes foregut development whereas, high levels repress.

Different thresholds of Fzd7/β-catenin regulate endoderm fate

The previous model of endoderm patterning predicted that "Wnt-ON" promotes hindgut and represses foregut fate whereas a "Wnt-OFF" state is required to specify foregut progenitors (McLin et al., 2007). Our data here indicate that this is an over simplification and suggests that endoderm progenitor development is controlled by multiple thresholds of Wnt/β-catenin signaling: (1) when β-catenin activity is reduced below a critical threshold, as in the Fzd7 morphants progenitor development is arrested; (2) in response to a low level of Wnt/β-catenin the endoderm cells adopt a foregut fate at the expense of hindgut endoderm fate; and (3) in response to a high level of Wnt/β-catenin, endoderm cells adopt a mid/hindgut fate and repress foregut fate. To more thoroughly test this hypothesis, we modulated both Fzd7 levels and β-catenin signaling in a progressive series of overlapping doses to determine if we could indeed generate embryos with each of the three predicted endoderm cell fates.

To stimulate a dose response of Wnt/β-catenin activity, we treated control or Fzd7-depleted sibling embryos from stage 10 to stage 20 with different concentrations of the small molecule BIO, which inhibits GSK3 and thus stabilizes β-catenin (Sato et al., 2004). In control un-manipulated stage 20 embryos, *hhex* and *vent1/2* are expressed in a reciprocal pattern, with *hhex* marking the foregut and *vent1/2* marking the mid/hindgut progenitors (Fig. 7C,D). As the dose of BIO (and therefore β-catenin activity) was increased, *hhex* was down regulated and *vent1/2* was ectopically expanded into the foregut domain (Fig. 7A); this indicates that the anterior endoderm has adopted a hindgut fate. We next progressively reduced Fzd7 levels by injecting different doses of the fzd7-MOs. Consistent with a multiple threshold model; partial knockdown of Fzd7 (25 ng of the fzd7-MOs) resulted in a modest expansion of *hhex* domain and modest down-regulation of *vent1/2* (Fig. 7E,F). In contrast, injection of 50 ng of the fzd7-MOs, which resulted in a more complete Fzd7 depletion caused a loss of *hhex* (Fig. 7G) as we have already shown in Fig. 2. Most importantly this loss of *hhex* in the complete Fzd7 knockdown was not accompanied by an expansion of *vent1/2* (Fig. 7H) as was seen when β-catenin activity was elavated (Fig. 7B); this suggests that foregut development was arrested rather than being re-specified to a hindgut fate. When we progressively added back β-catenin signaling to the 50 ng fzd7-MO injected embryos (via BIO treatment), we found that a low dose of BIO restored *hhex* expression (Fig. 7I) whereas a higher BIO dose once again repressed *hhex* and expanded *vent1/2* (Fig. 7K,L). We conclude from these experiments that different thresholds of Wnt/Fzd7/β-catenin signaling control endoderm progenitor fate in the *Xenopus* embryo.

Discussion

Thresholds of Wnt/Fzd7 signaling coordinate endoderm progenitor development

Previous studies suggested a model of *Xenopus* endoderm patterning where "Wnt-OFF" promotes foregut development and "Wnt-ON" specifies hindgut (Li et al., 2008; McLin et al., 2007). However, our results here support a revised model where multiple thresholds of Wnt/Fzd7/β-catenin and Wnt/Fzd7/JNK activity coordinate cell fate, proliferation and morphogenesis (Fig. 8). Our results shed light on the dynamic role of Wnt signaling during endoderm development and may help to resolve a number of disparate observations in the literature reporting differential effects of Wnt signaling on endoderm lineages (Goessling et al., 2008; Goss et al., 2009; Lade and Monga, 2011; Ober et al., 2006; Poulain and Ober, 2011).

Our findings here together with previous reports suggest that the high levels of Wnt/β-catenin signaling, which occur in the posterior, cause endoderm cells to adopt a hindgut fate and repress foregut identity. In the anterior endoderm the Wnt-antagonist Sfrp5 (Li et al., 2008) maintains Wnt/Fzd7/β-catenin activity at a low (but essential) threshold required to maintain foregut progenitors and repress hindgut fate. However, if β-catenin signaling is too low (as in Fzd7 morphants) endoderm progenitor development is blocked and proliferation is dramatically reduced (Fig. 8B).

With respect to Wnt/Fzd7/JNK signaling we propose that there may be differential activity between the deep and surface endoderm cells. In *Xenopus* the early endoderm is not a single cell layer sheet as in mouse but rather a mass of tissue approximately 15–20 cells thick. Our results together with previous studies suggested that Wnt/JNK activity is required in the deep endoderm (foregut and hindgut) for polarized cell movements and gut elongation (Li et al., 2008 and Supplementary Fig. S6). We demonstrate that JNK activity in the foregut endoderm requries Fzd7 and that when the threshold of Wnt/JNK activity is too low (as in the Fzd morphants) both the deep and surface endoderm cells exhibit an enlarged size, reduced adhesion and have a random orientation. On the other hand when JNK activity is too high, such as when caJNK is over expressed or when Sfrp5 is depleted (Li et al., 2008) cell morphology and adhesion is also disrupted. The observation that too much Wnt/JNK or too little Wnt/JNK can cause similar phenotypes has also been reported in other contexts (Kim and Han, 2005; Wallingford and Habas, 2005). Recent evidence suggests that Sfrps can exert biphasic concentration dependent activities; inhibiting Wnts at high concentration and facilitating Wnt signaling at low concentrations (Mii and Taira, 2009). We postulate that in foregut surface cells, which specifically express Sfrp5, Wnt/Fzd7/JNK activity is maintained at a low but essential threshold necessary to form an epithelium, and that diffusion of low levels of Sfrp5 protein into the deep foregut tissue may facilitate Wnt/Fzd7/JNK activity to promoting morphogenesis as well as maintain *hhex* expression and proliferation (Fig. 8C).

Fzd7 stimulates both Wnt/β-catenin and Wnt/JNK pathways to coordinate foregut cell identity, morphogenesis and proliferation

Fzd7 and its putative ligands in the foregut Wnt11 and Wnt5a, can stimulate either canonical Wnt or non-canonical Wnt transduction pathways depending on the cellular context (Cha et al., 2008; Medina et al., 2000; Mikels and Nusse, 2006; Sumanas and Ekker, 2001; Tao et al., 2005). However, there is little evidence that Wnt/Fzd signaling can activate both pathway simultaneoulsy in the same tissue; indeed in most instances the canonical and non-canonical branches appear to be mutually antagonistic (Grumolato et al., 2010; Topol et al., 2003). Our data indicate that in the *Xenopus* foregut Fzd7 activates both Wnt/β-catenin and Wnt/JNK pathways, which cooperate rather than antagonize each other to coordinate foregut progenitor proliferation and gene expression. Although we cannot rule out the possibility that different cells in the foregut activate β-catenin or JNK, our data suggest that these two pathways act in parallel rather that in a linear fashion since manipulation of one pathway did not appear to impact the activity of the other.

The fact that the Fzd7 morphant phenotype is distinct from the previous reported Wnt11 foregut-knockdown (Li et al., 2008) suggests that Fzd7 may interact with multiple, redundant Wnt ligands including Wnt5a, Wnt5b, Wnt8 and Wnt11. Interestingly maternal Wnt5a and Wnt11 are able to form heteromeric complexes to activate canonical signaling in the *Xenopus* blastula (Cha et al., 2009). Future studies will test whether different combinations of Wnt ligands signal through Fzd7 to elicit distinct downstream effects.

Fzd7/JNK regulation of cadherin and the cytoskeleton in the foregut

Wnt/JNK can regulate cell polarity, motility and the cytoskeleton in many contexts (Bovolenta et al., 2006; Kim and Han, 2005; Seifert and Mlodzik, 2007). Previous work suggests that Wnt/JNK activity is required in the deep endoderm for early gut elongation (Li et al., 2008) and consistent with this Fzd7 morphants exhibit a short gut. We postulate several possible mechanisms by which Fzd7/JNK-mediated signaling might influence foregut cell adhesion and the cytoskeleton:

1. Fzd7-mediated JNK activity might directly regulate the interaction between the actin cytoskeleton and cell adhesion complexes. For example in human primary keratinocytes JNK activity is required for the association of β-catenin to β-catenin/E-cadherin at adhesion junctions (Lee et al., 2011). If JNK were playing a similar role in the *Xenopus* foregut this might account for the altered cadherin localization and loss of cortical actin in Fzd7 morphants.
2. Alternatively the actin cytoskeleton could be the primary target of Fzd7 regulation. Non-canonical Wnt/Fzd signaling regulates small GTPases including Cdc42, Rho and Rac (Schlessinger et al., 2009), which can modulate JNK and regulate the formation of actin stress fibers, and these can in turn influence the localization of cadherins to nascent adhesion junction (Chu et al., 2004; Vaezi et al., 2002; Vasioukhin et al., 2000).
3. Fzd7 may also regulate the cadherin cycling to the membrane. In the zebrafish gastrula Wnt11/Fzd7 can influence cell cohesion by regulating E-cadherin

endocytosis via GTPase Rab5c (Ulrich et al., 2005). In addition Wnt11$^{-/-}$ mouse cardiomyocytes exhibit abnormal localization of N-cadherin, β-catenin and actin (Nagy et al., 2010), similar to Fzd7 morphants.

4. Finally it is possible that Fzd7 regulates the activity of other adhesion molecules such as proto-cadherins (Schambony and Wedlich, 2007) or Flamingo the apical cadherin Wnt/PCP co-receptor (Usui et al., 1999).

Conclusions

Using a foregut specific loss-of-function we demonstrate that Fzd7 mediates a low, but essential level of Wnt/β-catenin and Wnt/JNK signaling that is required for foregut development. Together with previous results our data support a model where Sfrp5-Wnt-Fzd7 interactions spatially regulate different thresholds of Wnt/β-catenin and Wnt/JNK signaling that coordinate endoderm progenitor fate, proliferation and morphogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Frizzled 7 is required for Xenopus foregut development
- Thresholds of Wnt/Fzd7 signaling pattern the endoderm progenitors
- Fzd7 signals via both the b-catenin and JNK pathways
- Fzd7 coordinates cell identity, proliferation and morphology

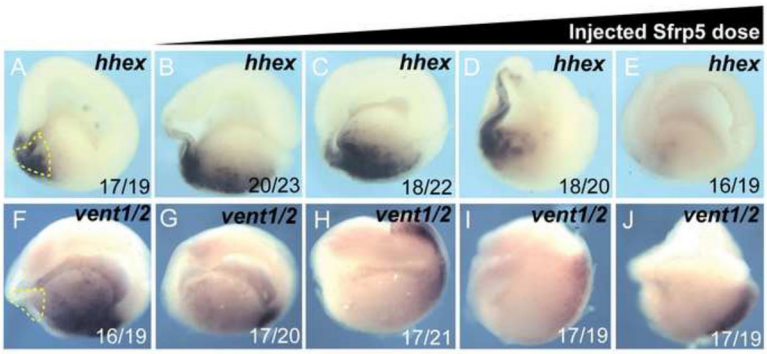

Figure 1. Differential Wnt signaling patterns the *Xenopus* endoderm

Wnt signaling has different thresholds in the endoderm. Embryos were injected with a dose range of mRNA encoding Sfrp5; 500 pg (B,G), 800 pg (C,H), 2 ng (D,I) and 3 ng (E,J). In situ hybridization for *hhex* (**A–E**) and *vent1* and *vent2*, referred to as *vent1/2* (**F–J**) in stage 19 bisected embryos showed that low doses of Sfrp5 expanded the *hhex* expression domain (yellow dashed line) (**B–D**) at the expense of hindgut markers *vent1* and *vent2* (a mixture of both probes referred to as *vent1/2*) (**G–I**). The highest dose of Sfrp5 resulted in a loss of *hhex* (**E**). The number of embryos with the illustrated phenotype is indicated in each panel.

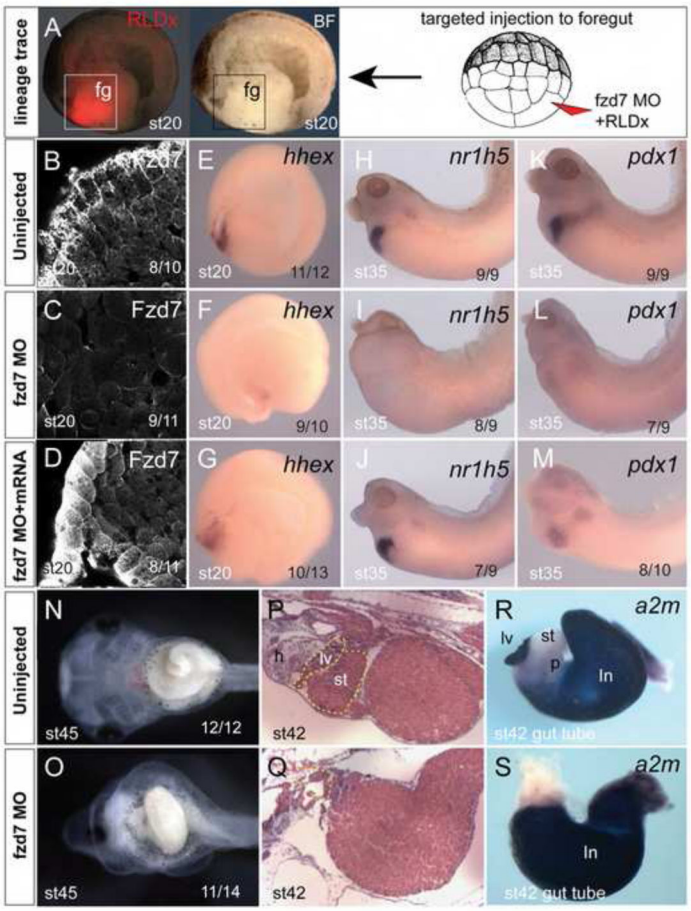

Figure 2. Fzd7-depletion disrupts foregut organogenesis

(**A**) Targeted injection of fzd7-MOs (50 ng) and red fluorescent tracer (RLDx) into the D1 cells of 32-cell stage *Xenopus* embryos. Bight field (BF) and fluorescent view of representative bisected stage 20 embryo showing that the injection targeted the foregut (fg) and avoided the axial mesoderm. (**B–D**) Confocal immunostaining confirmed that the fzd7-MOs resulted in a loss of Fzd7 protein from the foregut cell membrane (**B,C**), which was rescued by injection of Fzd7 mRNA lacking MO-target sequence (**D**). (**E–M**) In situ hybridization showed that fzd7-MO embryos failed to maintain *hhex* at stage 20 and did not express the liver (*nr1h5*) and pancreas/duodenum (*pdx1*) markers at stage 35, which could be partially rescued by injection of Fzd7 mRNA. (**N, O**) Ventral view of stage 45 embryos showing foregut edema and defective intestinal coiling with shortened gut in fzd7-MO injected embryos. (**P,Q**) H&E-stained section of a control (**P**) and a fzd7-MO-injected embryo (**Q**) that lacks foregut organs including liver (lv) and stomach (st). (**R, S**) In situ hybridization of *a2m* in isolated stage 42 gut tubes showed loss of foregut organs such as liver (lv), stomach (st) and pancreas (p) and shortened intestine (In). The number of embryos with the illustrated phenotype is indicated in each panel.

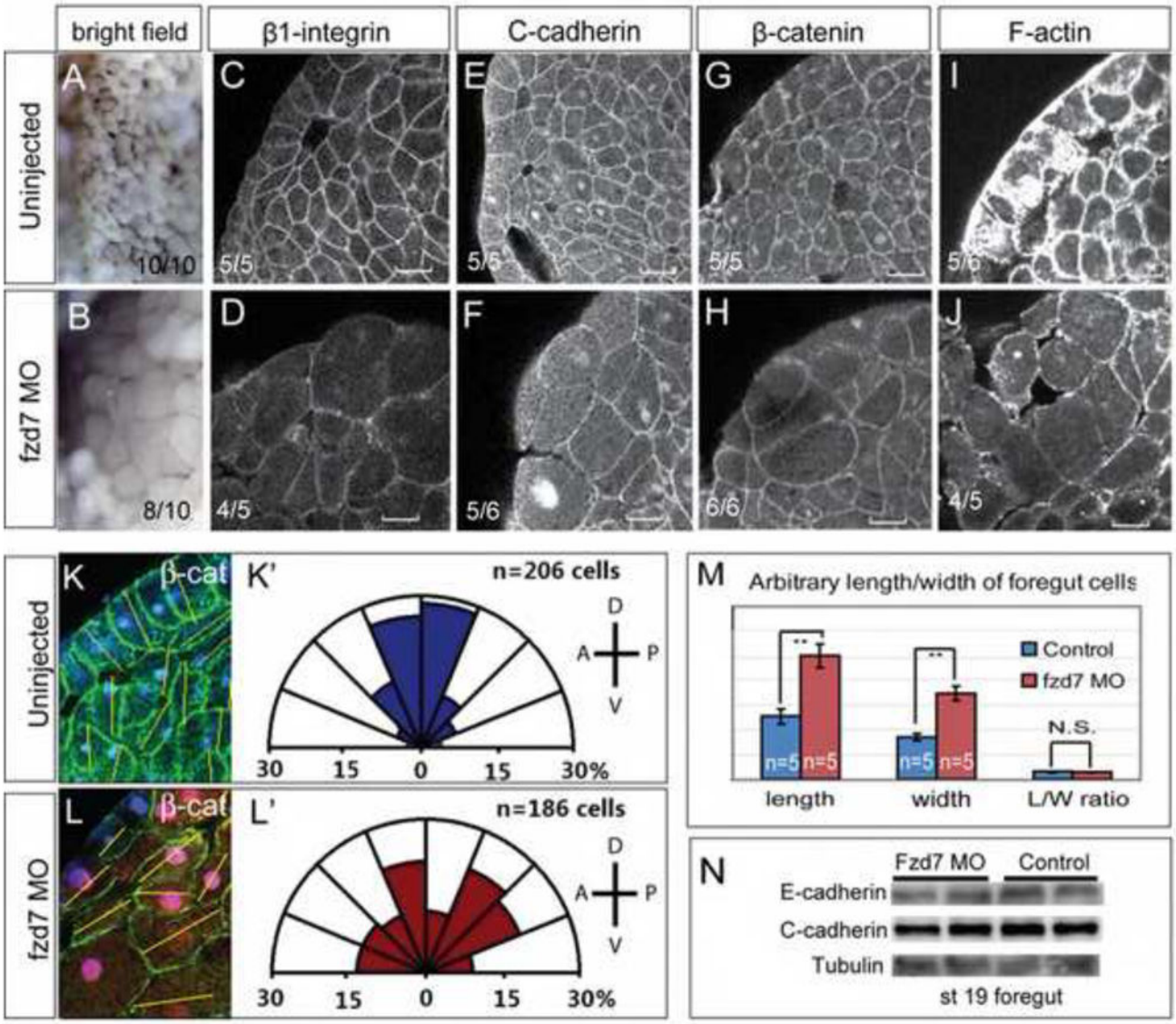

Figure 3. Fzd7-depletion causes defects in foregut cell morphology

(**A–B**) Bight field view of the foregut surface at stage 20 showed that Fzd7 morphants exhibit larger loosely adherent cells (**B**), compared to controls (**A**). (**C–J**) Confocal immunostaining of the foregut (anterior left, dorsal up) with µ1-integrin (**C,D**), C-cadherin (**E,F**), β-catenin (**G,H**) and phalloidin (F-actin) (**I,J**) showed decreased cell adhesion molecules and reduced cytoskeleton in the enlarged foregut cells of Fzd7 morphants. All of the images were taken using same setting for control and fzd7-MO embryos. (**K–L**) Quantitation of cell size and orientation; foregut cell length, width and orientation in control (**K**) and fzd7-MO injected embryos (**L**) were measured from β-catenin immunostaining (green) using Image-J. Nuclei shown in blue, fzd7-MO/RLDx in red. All images were oriented with dorsal up. The yellow line marks the long axis of foregut cells, and quantification shows the frequency of orientations of the long axis of cells in Fzd7 morphants (**L,L′**) and control **(K,K′**) n= the total number of foregut cells from 5 uninjected (K′) and 5 fzd7-MO embryos (L′). (**M**) The relative length, width, and length/width radio in

control and Fzd7-depleted foregut cells. **p<0.01 relative to controls in Student's t-test (**N**) Western blot analysis shows no significant changes of total E-cadherin and C-cadherin level in the foregut explants.

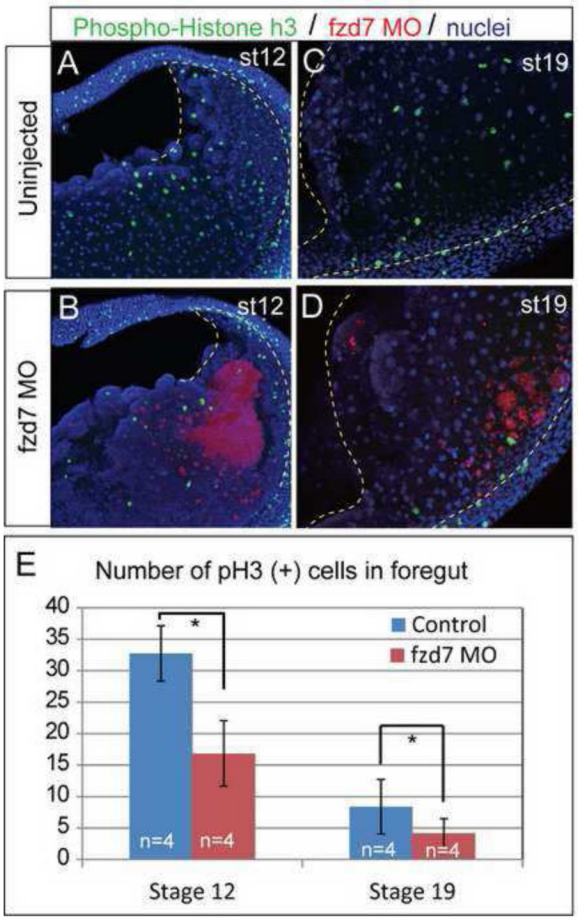

Figure 4. Fzd7-depletion causes reduced cell proliferation

(**A–D**) Confocal immunostaining of phospho-Histone h3 (PH3; green), nuclei (blue) and fzd7-MO/RLDx (red) at stage 12 (**A,B**) and stage 19 (**C,D**) show that Fzd7-depleted embryos exhibit reduced foregut (outlined in dashed yellow line) proliferation. (**E**) Mean number of PH3 positive cells in the foregut +/− S.D. *p<0.05 relative to sibling controls in Student's t-test (n= 4 embryos/condition).

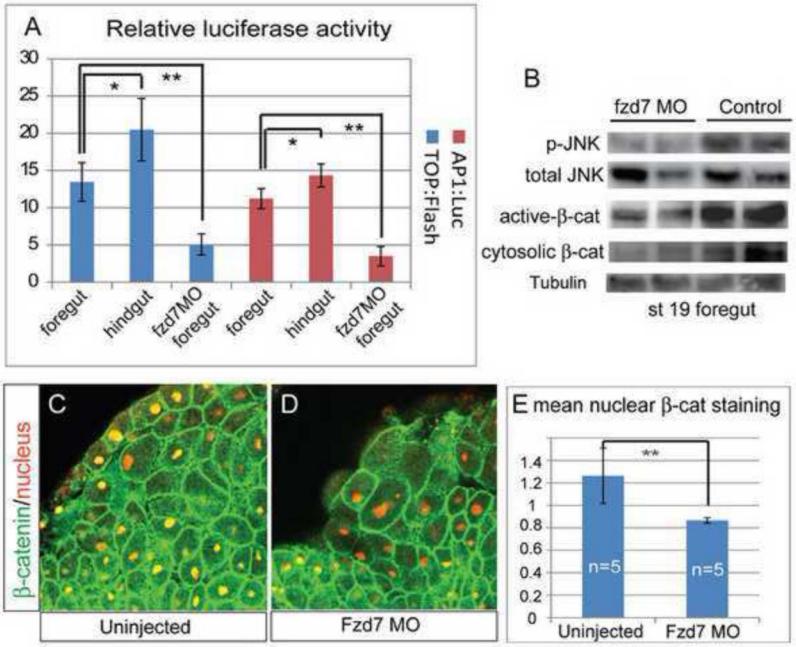

Figure 5. Fzd7 depletion results loss of both Wnt/β-catenin and Wnt/JNK activity in the foregut

(**A**) Fzd7-depletion resulted in a reduction of β-catenin/Tcf and JNK/AP1 activity in the foregut. TOP:flash or AP1:Luciferase reporter plasmids were injected into either the D1 foregut endoderm cells or the D4 hindgut endoderm cells at the 32-cell stage, with or without fzd7-MO as indicated. The TOP:Flash reporter is an indicator of β-catenin/Tcf activity, while the AP1:luciferase reporter is an indicator of JNK-mediated c-Jun/c-Fos (AP1) activity. At stage 20 luciferase activity was measured, in triplicate. The average relative luciferase activity, normalized to co-injected pRTK:Renila, from three biological replicates per condition is shown +/− S.D. *$p<0.05$ and ** $p<0.01$ relative to control foregut in Student's t-test. (**B**) Western blot showed decreased phospho-JNK1/2 (p-JNK) and a loss of dephosphorylated active β-catenin and total cytosolic β-catenin in the foregut explants at stage 19. (**C–D**): Confocal immunostaining showed reduced nuclear β-catenin levels in Fzd7 morphant foregut tissue (**D**) relative to controls (**C**), at stage 20. (**E**) Mean pixel intensity of nuclear β-catenin staining measured using Image-J +/− S.D (foregut cells were scored from 5 embryos/condition).

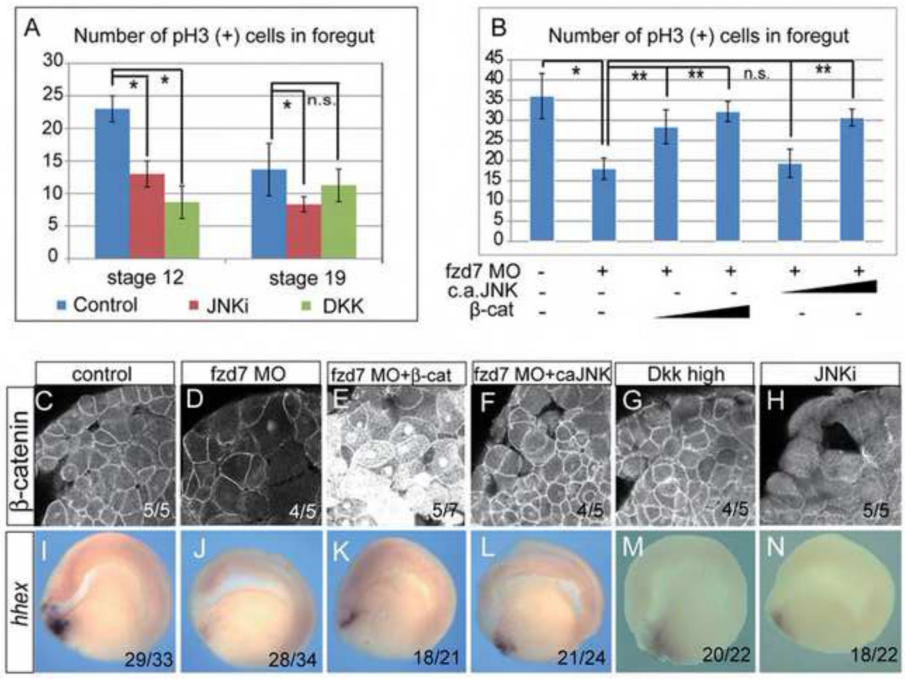

Figure 6. Fzd7 signals via both β-catenin and JNK coordinate foregut cell proliferation, gene expression and cell morphology

(**A**) Inhibition of either Wnt/β-catenin or JNK pathways reduced foregut cell proliferation. Embryos were either injected with RNA encoding Dkk1 (500 pg) to block the Wnt/β-catenin pathway or treated with the cell soluble JNK inhibitor SB600125 (JNKi; 100 μM). Mean number of PH3 positive cells in the foregut +/− S.D. *p<0.05 and ** p<0.01 compared to controls (n=4 embryos/condition). (**B**) Activation of either β-catenin or JNK signaling rescued cell proliferation in Fzd7 morphants. Embryos were injected with fzd7-MOs (50 ng) with or without RNA encoding a constitutively active JNK (c.a.JNK; 200 pg) or a hormone inducible GR:Lef-βCTA (β-cat; 200 pg) activated by 1 μM dexamethasone at stage 11. Mean number of P-H3 positive foregut cells at stage 12 +/− S.D. *p<0.05 relative to control and **p<0.05 relative to fzd7-MO alone in Student's t-test (n= 4 embryos/condition). (**C–H**) Fzd7/JNK signaling regulates cell shape. Confocal immunostaining of β-catenin at stage 20 showed that c.a.JNK injection (**F**) rescued the cell-size defects in Fzd7 morphants (**D**), whereas activation of the GR:Lef-βCTA (β-cat) did not (**E**). The JNK inhibitor (JNKi) caused a reduction of cytoskeletal β-catenin and increased cell size (**H**), phenocopying fzd7-MO (**D**), whereas Dkk1 (1.5 ng) had no effects on cell morphology (**G**). (**I–N**) Both Fzd7/β-catenin and Fzd7/JNK regulate gene expression. In situ hybridization to stage 20 embryos showed that co-injection of either GR:Lef-βCTA (β-cat) (**K**) or c.a.JNK (**L**) restored *hhex* expression in Fzd7 morphants (**J**), whereas the JNK inhibitor (**N**) or high levels of Dkk1 (1.5 ng) (**M**) suppressed *hhex*. The number of embryos with the illustrated phenotype is indicated in each panel.

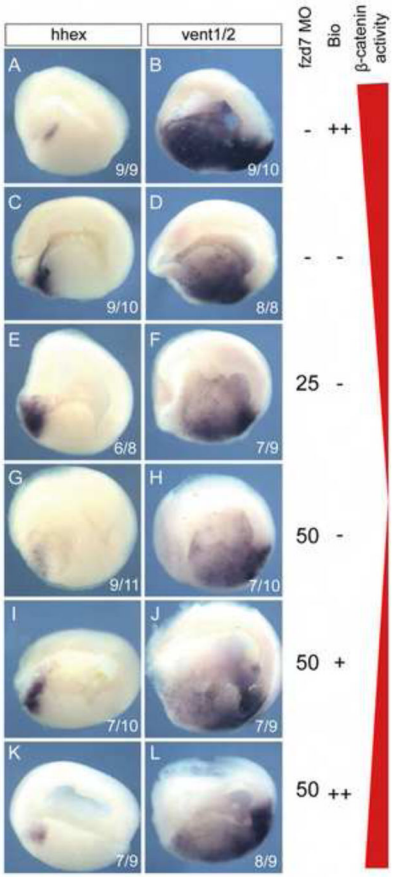

Figure 7. Multiple thresholds of Wnt/Fzd7/β-catenin activity pattern the endoderm

(**A**–**L**) Fzd7/β-catenin signaling levels were modulated in a dose response with different combination of fzd7-MOs and/or treatment with BIO, a GSK3 inhibitor that stabilizes β-catenin. In situ hybridization of the foregut marker *hhex* and the mid/hindgut markers *vent1/2* in stage 20 embryos showed that increasing dose of BIO and therefore increasing β-catenin activity decreased *hhex* while expanding *vent1/2* (**A, B**) relative to untreated controls (**C, D**). A partial knockdown of Fzd7 (25 ng of fzd7-MOs) resulted in a modest increase in *hhex* and reduction of *vent1/2* (**E, F**), whereas a complete Fzd7 knockdown by 50 ng of the fzd7-MO caused a loss of *hhex*, which was not accompanied by an expansion of *vent1/2* (**G, H**). A low dose of BIO (5 uM in I,J) rescued *hhex* expression in fzd7-depleted embryos (**I, J**), whereas a higher BIO dose (10 uM) resulted in the foregut adopting a hindgut fate and expressing ectopic *vent1/2* (**K, L**). The number of embryos with the illustrated phenotype is indicated in each panel.

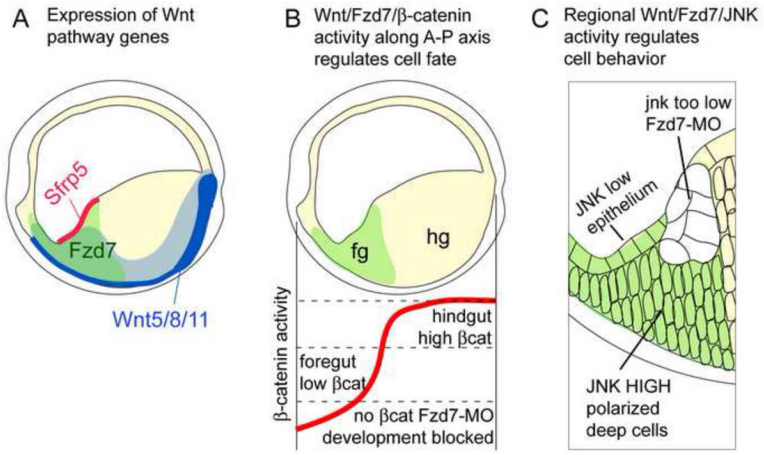

Figure 8. A model of how Wnt/Fzd7/Sfrp5 regulate β-catenin and JNK signaling to coordinate endoderm fate, morphogenesis

(**A**) Schematic of a neurula embryo (anterior left) showing expression of the *fzd7* (green), *wnt11*, *wnt5* and *wnt8* in the ventral mesoderm and endoderm (blue) and the Wnt-antagonist *sfrp5* in the surface of the foregut endoderm (red). The spatial expression pattern of receptors, ligands and antagonists is postulated to establish differential Wnt activity in the endoderm. (**B**) Different thresholds of β-catenin/TCF activity (red line) pattern endoderm with high activity promoting hindgut progenitor fate (hg), where as a low but essential level of β-catenin/TCF is required to maintain foregut (fg) fate. (**C**) Differential Fzd7/JNK activity might regulate cell shape, adhesion and morphogenesis in the foregut (green). Fzd7/JNK signaling in the deep endoderm promotes cell adhesion and the oriented cell shape required for gut elongation. Sfrp5 in the surface layer reduces JNK activity to a low but essential level to establish an epithelium. If JNK activity is too low (as in Fzd7 morphants) cells become enlarged, loosely adherent with a random orientation.