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Syndecan4 coordinates Wnt/JNK and BMP signaling to regulate foregut progenitor development

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Abstract

Temporally and spatially dynamic Wnt and BMP signals are essential to pattern foregut endoderm progenitors that give rise to the liver, pancreas and lungs, but how these two signaling pathways are coordinated in the extracellular space is unknown. Here we identify the transmembrane heparan sulphate proteoglycan Syndecan-4 (Sdc4), as a key regulator of both non-canonical Wnt and BMP signaling in the *Xenopus* foregut. Foregut-specific Sdc4 depletion results in a disrupted Fibronectin (Fn1) matrix, reduced cell adhesion, and failure to maintain foregut gene expression ultimately leading to foregut organ hypoplasia. Sdc4 is required to maintain robust Wnt/JNK and BMP/Smad1 signaling in the *hhex*+ foregut progenitors. Pathway analysis suggests that Sdc4 functionally interacts with Fzd7 to promote Wnt/JNK signaling, which maintains foregut identity and cell adhesion. In addition, the Sdc4 ectodomain is required to support Fn1 matrix assembly, which is essential for the robust BMP signaling that promotes foregut gene expression. This work sheds lights on how the extracellular matrix can coordinate different signaling pathways during organogenesis.

Keywords

Syndecan; Extracellular matrix; Fibronectin; Fzd; Wnt; BMP; Foregut; Progenitor; Endoderm; *Xenopus*; *hhex*

1. Introduction

The endoderm germ layer gives rise to the epithelial lining of the digestive and respiratory tracts and associated organs such as liver, pancreas, and lungs. Shortly after gastrulation a series of growth factor signals from adjacent mesoderm progressively patterns the naïve embryonic endoderm along the anterior-posterior (A–P) axis and to induce various organ lineages in the primitive gut tube (Horb and Slack, 2001; Zaret, 2008; Zorn and Wells, 2009). These paracrine signals are highly dynamic, with the same factors having dramatically different impacts on organogenesis at different times or at different positions along the embryonic gut tube (McLin et al., 2007; Wandzioch and Zaret, 2009). The mechanisms that coordinate these signaling dynamics *in vivo* are poorly understood.

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Previous studies in *Xenopus* have shown that precise levels of Wnt and BMP during late gastrula and early somite stages are critical to pattern and maintain foregut versus hindgut progenitors. At this stage in development a combination of low-Wnt and high-BMP are required to maintain the ventral foregut progenitors, which express the homeobox gene *hhex*, whereas high-Wnt and high-BMP promotes hindgut progenitors (Kenny et al., 2012; McLin et al., 2007). Differential Wnt activity is controlled in part by the secreted Wnt-antagonist Sfrp5, which protects anterior endoderm from posterior Wnt ligands (Li et al., 2008). As a result the foregut cells experience a low, but critical level of both Wnt/β-catenin and Wnt/JNK signaling through Wnt receptor the Frizzled 7 (Fzd7) to coordinate foregut identity and morphogenesis (Zhang et al., 2013). If Wnt activity is too high, such as with the loss of Sfrp5 or Wnt overexpression, the anterior endoderm adopts a hindgut rather than foregut fate (Li et al., 2008; McLin et al., 2007). On the other hand, if Wnt activity in the foregut is too low, such as in Fzd7 depleted embryos, the foregut progenitors arrest with disrupted cell morphology and fail to maintain *hhex* expression resulting in foregut organ agenesis (Zhang et al., 2013).

During the same period of development, BMP ligands secreted from the cardiac mesoderm are required to maintain ventral foregut progenitors in *Xenopus*. Recent studies indicate that this BMP signaling is dependent upon the extracellular Fibronectin (Fn1) matrix that forms between the endoderm and the adjacent mesoderm (Kenny et al., 2012). Current evidence suggests that a functional balance between secreted Tolloid-like (Tll) metalloproteinases and the Tll-inhibitor Sizzled (Szl) modulate the de-position of Fn1 fibrils and that the Fn1-rich extracellular matrix (ECM) promotes robust BMP signaling, perhaps by increasing the local ligand concentration in the foregut microenvironment, which is critical for liver, pancreas, and lung organogenesis (Kenny et al., 2012).

Exactly how the ECM coordinates Wnt and BMP activity in the foregut is still poorly understood. Although genetic and bio-chemical studies (primarily in *Drosophila* and cell culture) indicate that the ECM is an important regulator of growth factor bioavailability and may provide a co-receptor function; in most cases the *in vivo* molecular mechanisms are still obscure (Hacker et al., 2005; Lin, 2004). In particular the role of Fn1, a vertebrate specific ECM protein, is still poorly defined. The transmembrane heparan sulphate proteoglycan (HSPG) Syndecan 4 (Sdc4) has emerged as a candidate for coordinating extracellular Wnt and BMP signaling in the *Xenopus* foregut. Sdc4 together with Integrin-α5β1 are well known to promote Fn1 matrix assembly and are key focal adhesions components that recruit other ECM proteins (Dzamba et al., 2009; Morgan et al., 2007; Ramirez and Rifkin, 2009; Schwarzbauer and DeSimone, 2011). In some tissues Syndecans act as co-receptors for various growth factors and can transduce signals *via* their intracellular domains (Alexopoulou et al., 2007). Notably Sdc4-Fn1 complexes can function as Fzd7 co-receptors to promote non-canonical Wnt signaling *via* Jun N-terminal Kinase (JNK) and Rho family of small GTPases in certain cellular contexts including: *Xenopus* gastrulation, neural crest migration and murine muscle cell homeostasis (Bentzinger et al., 2013; Davidson et al., 2006; Escobedo et al., 2013; Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011).

In this study we tested the hypothesis that Sdc4 might coordinate Fn1-regulated Wnt and BMP signaling in the *Xenopus* embryonic foregut. Using foregut specific depletion we show that Sdc4 is required to maintain foregut progenitor identity, proliferation and morphogenesis. Our data suggest that Sdc4 works together with Fzd7 to transduce Wnt/JNK signals that maintain foregut gene expression and cell-cell adhesion. In addition, Sdc4 is required for Fn1 matrix deposition at the boundary between foregut endoderm and BMP-expressing cardiac mesoderm. Loss of the Fn1 matrix in Sdc4-depleted embryos disrupts a positive BMP-feedback loop that is critical for maintaining the foregut progenitors. Together these data provide a paradigm for how Sdc4 regulation of the ECM might coordinate Wnt and BMP signaling in many development and disease contexts.

2. Material and methods

2.1. Embryo manipulations and microinjections

Xenopus laevis embryo manipulations were performed as previously described (McLin et al., 2007) and staged based on the Nieuwkoop and Faber normal table of development (Nieuwkoop, 1994). To specifically target the foregut endoderm and avoid the chordomesoderm, antisense morpholino oligos (MO) and/or mRNAs (along with a fluorescent lineage tracer) were micro-injected into D1 cells of 32-cell stage embryos (Moody, 1987). To knockdown Sdc4, previously validated antisense MOs were used at either a total amount of 25 or 50 ng (Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011). Other MOs were: Fzd7-MOs (25 or 50 ng) (Sumanas and Ekker, 2001); Szl-MO (20 ng) (Collavin and Kirschner, 2003; Lee et al., 2006), Fn1 MOs:Fn1.S-MO + Fn1. L-MO (25 ng each) (Davidson et al., 2006) and Cdh3 MO (Ninomiya et al., 2012). For all experiments the total amount of MO injected is equalized by addition of the standard control MO (GeneTools) as necessary. Synthetic mRNA was generated from the following plasmids: pCS2+Sdc4 (Munoz et al., 2006), pCS2+Sdc4ΔPBM (kindly provided by Dr. J. Larrain) (Carvallo et al., 2010), and pCS2+c.a.JNK (Liao et al., 2006). Recombinant hBMP2 (10 μM, 15 nl; R&D Systems) or Wnt11 protein (10 μM, 20 nl; R&D Systems) was injected into the closing blastocoel at stage 13. The following cell-soluble inhibitors were dissolved in DMSO and added to the media at stage 12: BMP-inhibitor LDN 193189 (100 μM, Axon Medchem), FGF-inhibitor PD173074 (300 μM; TOCRIS), and FGF-inhibitor SU5402 (10 μM with 0.1 M ATP). All injection and inhibitor experiments were repeated at least three independent times with similar results and a representative example is shown.

2.2. In situ hybridization and immunohistochemistry

In situ hybridization and confocal immunohistochemistry were performed as previously described (Zhang et al., 2013). Details on the antisense RNA probes used are available upon request. The following primary antibodies were used: rabbit anti-β-catenin (1:250; H-102, Santa Cruz Biotechnologies), mouse anti-Cdh3 (1:200; 6B6, DSHB), mouse anti-β1-Integrin (1:500; 8C8, DSHB), rabbit anti-phospho-HistoneH3 (1:250; Cell signaling), rabbit anti-active-JNK (1:250; Promega), anti-Fibronectin (1:250; 4H2; gift from Dr. DeSimone) and rabbit anti-phospho-Smad 1/5/8 (1:250; Cell signaling). 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.

Image-J was used to quantify the pixel intensity of nuclear pSmad1/5/8, pJNK and nuclear β-catenin immunostaining in the MO-targeted foregut endoderm cells (50–150 cells per foregut), using 15 mm mid-sagittal optical sections of five stage 19 embryos for each condition. A fluorescent lineage tracer was used to identify the injected cells and Topro-3 staining was used to define the nucleus from surrounding cytoplasm and to normalize the antibody intensity. The average normalized pixel intensity (in arbitrary units) was calculate for each condition +/− standard deviation. Repeated measure ANOVA with covariance structure using R software was used to assess statistically significant differences in mean pSmad1/5/8, pJNK and nuclear β-catenin intensity between the foregut endoderm cells in different condition compared to controls. To calculate the average mitotic index at stage 11, we count the total number of phospho-Histone H3 positive nuclei in the anterior endoderm, divided by total number of anterior endoderm cells (~100 cells) or-# for each experimental groups (n=4 embryos/condition). All experiments were repeated three times with similar results.

2.3. BRE:Luc, TOP:flash and ATF2:luciferase assay

BMP/pSmad1, Wnt/β-catenin and Wnt/JNK activity in the foregut were assayed using transcriptional reporter assays. BRE: Luciferase (150 pg) (von Bubnoff et al., 2005), Top-flash (150 pg) (Korinek et al., 1997) or ATF2:luciferase, plasmids (van Dam et al., 1995) co-injected with pRL-TK:renilla (25 pg) into D1 presumptive foregut cells of 32-cell stage embryos. At stage 19 extracts were prepared from each condition 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. with pairwise student *t*-tests to determine significant differences in expression. Each experiment was repeated a minimum of three times and a representative result is shown.

2.4. Western blot

Western blots of dissected foregut tissue was carried out as previously described (Cha et al., 2008). Antibody concentrations were rabbit anti-Sdc4 (1:500; Imgenex); rabbit anti-β-catenin (1:500; H-102, Santa Cruz Biotechnologies), mouse anti-Cdh3 (1:500; 6B6, DSHB), mouse anti-β1-integrin (1:500; 8C8, DSHB), mouse anti-Cdh1 (1:500; 5D3; DSHB); mouse anti-Fibronectin (1:500; 4H2; gift from Dr. DeSimone) and mouse anti-Tubulin (1:5000; Neomarker).

3. Results

3.1. Sdc4 is required for foregut organogenesis

Previous studies have shown that *sdc4* transcripts are robustly expressed in ectoderm, anterior endoderm, and the involuting chordomesoderm of *Xenopus* gastrulae, where Sdc4 is required for non-canonical Wnt/Fzd7-mediated gastrulation movements (Munoz et al., 2006). To explore the possibility that Sdc4 may have a role in foregut development we

performed *in situ* hybridization. *sdc4* was expressed in the foregut endoderm from the late gastrula (NF11) to 20 h postfertilization (hfp) stages NF19 (Fig. 1B and Supplemental Fig. S1); a time in development when the foregut is experiencing both Wnt and BMP signaling.

Gastrulation defects caused by global Sdc4 knockdown preclude analysis of later organogenesis (Munoz et al., 2006). Thus to test the role of Sdc4 specifically in the foregut we injected well-characterized antisense Sdc4 morpholino oligos (Sdc4-MO) (Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011) into the D1 cells of 32-cell stage embryos, which give rise to the ventral foregut endoderm (Moody, 1987). Co-injection of a fluorescent lineage tracer confirmed that the D1-injected Sdc4-MO was largely restricted to the foregut (Fig. 1C) and did not result in obvious gastrulation defects. In contrast injection into the B1 cells that target the chordomesoderm resulted in severe disruptions to gastrulation as previously described, and these embryos died by NF35 (Supplemental Fig. S2).

Western blot analysis of dissected foregut tissue from control embryos at stage NF19 showed multiple bands approximately 30kD in size consistent with Sdc4 and its post-translationally modified isoforms as previously described (Munoz et al., 2006; Morgan et al., 2007; Astudillo et al., 2014). Foregut injection of the Sdc4-MO resulted in a knock down of approximately 75% (Fig. 1D).

At 96 hpf (stage NF45), when the GI tract becomes functional, Sdc4-MO embryos exhibited dramatic gut defects including edema, foregut organ hypoplasia, and disrupted gut coiling (Fig. 1E and F). In-situ hybridization of isolated gut tubes at 80 hpf (NF42) detecting *alpha-2-macroglobulin* (*a2m*), a marker of the liver and intestine, confirmed the lack of a liver bud as well as hypoplastic pancreatic buds (Fig. 1H and I). Earlier in development, at 50 hpf, (NF35) when organ lineages are first specified, liver (*nr1h5*) and pancreas (*pdx1*) markers were largely absent in Sdc4 morphants (Fig. 1L and O) indicating a failure of lineage specification. To confirm that the phenotype was due to loss of Sdc4, we sequentially injected the Sdc4-MO followed by a synthetic *sdc4* mRNA lacking MO-target sequence (Munoz et al., 2006), which restored liver and pancreatic gene expression and morphology (Fig. 1G, J, M and P). We conclude that Sdc4 is required for *Xenopus* foregut organogenesis.

3.2. Sdc4 and Fzd7 are required to maintain foregut gene expression and proliferation

To better understand why Sdc4 depletion disrupts foregut organogenesis we examined expression of the homeobox gene *hhex*, one of the earliest markers of foregut fate (Fig. 2A–L). *In situ* hybridization of Sdc4-MO embryos revealed a modest reduction of *hhex* in the anterior endoderm as early as mid-gastrula (NF11) (Fig. 2B), and by 20 hpf (NF19) *hhex* was almost undetectable (Fig. 2G), indicating a failure to maintain foregut progenitor identity. Importantly, injection of *sdc4* mRNA rescued *hhex* expression in Sdc4-MO embryos (Fig. 2C and H).

The loss of *hhex* and foregut hypoplasia in Sdc4-MO embryos was strikingly similar to what we previously observed when Fzd7 was depleted from the foregut (Fig. 2J, Zhang et al., 2013). The similar phenotypes, along with published reports that Sdc4 and Fzd7 can

physically interact to transduce non-canonical Wnt signals in other cellular contexts (Astudillo and Larrain, 2014), suggested that Sdc4 and Fzd7 might work together to promote *hhex* expression. To test this we co-injected into the presumptive foregut lower doses of Sdc4-MO (25 ng) and Fzd7-MO (25 ng), which individually did not cause any detectable phenotype (Fig. 2F and I). The low dose co-injection of both Sdc4-MO and Fzd7-MO resulted in reduced *hhex* similar to the 50 ng high dose injection of either knockdown separately (Fig. 2K and Supplemental Fig. S2). This experiment suggests that Sdc4 and Fzd7 work together to regulate foregut development.

We had previously shown that Wnt/Fzd7 signaling was critical to maintain proliferation of foregut progenitors (Zhang et al., 2013). Therefore we performed phospho-Histone H3 (pH3) immunostaining to identify mitotic cells. Sdc4 depletion resulted in significantly lower proliferation rate in the anterior endoderm cells at the late gastrula stage (NF11), similar to Fzd7-depleted embryos (Fig. 2M). By NF19 the Sdc4-MO and Fzd7-MO embryos had on average ~50% fewer cells in the foregut region compared to controls. The reduced cell proliferation in Sdc4-MO embryos was partially rescued by injection of synthetic *sdc4* mRNA (Fig. 2M). Together these data indicate that both Sdc4 and Fzd7 are required between NF10–20 to maintain *hhex* expression and proliferation in early foregut progenitors.

3.3. Sdc4 and Fzd7 regulate foregut cell adhesion and morphology

Sdc4-depleted foregut cells were larger than controls, disorganized and loosely adherent to one another at NF19, reminiscent of the phenotype caused by loss of non-canonical Fzd7/JNK activity in foregut (Zhang et al., 2013). To investigate this in more detail we examined proteins involved in cell adhesion and the cortical cytoskeleton. Immunostaining showed reduced cortical β-catenin localization in the Sdc4-MO foregut cells similar to Fz7 depletion (Fig. 3A–H). Western blot analysis of dissected foreguts revealed that total β-catenin (Ctnnb1) and Cadherin 1 (Cdh1; E-cadherin) levels were unaltered in Sdc4-depleted foreguts. However Sdc4-MO foreguts exhibited reduced Cadherin 3 (Cdh3; C-cadherin) and modestly lower Integrin beta 1 (Itgb1) (Fig. 3I). Consistent with the Western data, Sdc4-MO foregut cells exhibited reduced Cdh3 and Itgb1 immunostaining at NF19 (data not shown). Analysis of earlier time points indicated that Cdh3 staining was cell-autonomously reduced in Sdc4-MO anterior endoderm cells as early as the mid-gastrula (NF11) (Fig. 3L–N).

Sdc4 depleted embryos had about half as many foregut cells as controls (60±10.5%, p=0.006), consistent with the reduced proliferation. In addition the foregut cells in Sdc4-MO and Fzd7-MO depleted embryos were larger (Fig. 3J) and more round with a random orientation compared to control-MO foregut cells, which are organized in polygonal arrays with the long axis of the cells oriented along the dorsal-ventral axis (Fig. 3K, Zhang et al., 2013). Similar to the gene expression and proliferation defects, the cell morphology phenotypes were (1) rescued by *sdc4* mRNA injection, and (2) could be caused by the low dose co-injection of Fzd7-MOs (25 ng) and Sdc4-MOs (25 ng) which individually had no phenotype (Fig. 3A–H).

To evaluate whether reduced Chd3 could account for the Sdc4-MO phenotype, we injected a Cdh3-MO (Ninomiya et al., 2012) into D1 cells at 32-cell stage and analyzed foregut

morphology and gene expression at 20 hfp (NF19). Immunostaining confirmed the loss of Cdh3, which as expected was accompanied by a cell autonomous reduction in cortical β-catenin (Supplemental Fig. S3A–D). Although the Cdh3-MO foregut cells were loosely adherent and somewhat disorganized, they were not enlarged like in Sdc4 or Fzd7 knockdowns. Moreover *hhex* expression was largely unaffected in Cdh3-MO (Supplemental Fig. S3), suggesting that loss of Cdh3 alone cannot account for all of the defects Sdc4-MO in foreguts.

Since Sdc4-Integrin complexes can signal *via* Focal adhesion kinase (FAK), we also considered the possibility that reduced integrin signaling could account for the phenotype. However, there was no obvious difference in phospho-FAK immunostaining between control and Sdc4-MO injected foregut cells (data not shown), suggesting that the remnant Sdc4 levels or another Syndecan family member is sufficient to support integrin signaling. We conclude that Sdc4 and Fzd7 coordinately regulate foregut progenitor cell adhesion and morphology, in part by modulating Cdh3 levels.

3.4. Sdc4 is required for Fn1 extracellular matrix deposition in the foregut

One possible explanation for the Sdc4-MO phenotype was a disruption to the foregut ECM, which might then impact cell-cell signaling. Since Sdc4, Itgb1 and cadherins are all involved for Fn1 matrix assembly (Schwarzbauer and DeSimone, 2011), we evaluated the Fn1 ECM in Sdc4-depleted foregut tissue. As previously described, confocal immunostaining of control foreguts at 20 hpf (NF19) reveals two prominent Fn1 fibril layers; one between ectoderm and mesoderm, and the other between mesoderm and foregut endoderm, with a peri-cellular Fn1 matrix also present surrounding the deep foregut endoderm cells (Fig. 4A, Kenny et al., 2012). In Sdc4-MO foreguts the peri-cellular Fn1 and the Fn1 layer between mesoderm and endoderm was largely absent (Fig. 4B). Western blot analysis showed that total Fn1 levels were not obviously altered in Sdc4-MO foregut tissue (Fig. 4D), consistent with Sdc4 being required for matrix assembly, but not Fn1 expression. In contrast, the Fn1 matrix appeared largely normal in Fzd7-MOs embryos (Fig. 4C). Thus we conclude that in the foregut Sdc4 is required for Fn1 matrix formation independently of Fzd7 signaling.

3.5. Sdc4 is required for both Wnt/JNK and BMP/Smad signaling in the foregut

We next examined whether the loss of Sdc4 impacted cell signaling in the foregut. A recent study has suggested that in addition to acting as a Fzd7 co-receptor to promote Wnt/JNK activation, Sdc4-Fn1 complexes might also inhibit Wnt/β-catenin signaling in some contexts (Astudillo et al., 2014). This was intriguing because in the *Xenopus* foregut Fzd7 transduces a low, but indispensable level, of both β-catenin and JNK activity; if β-catenin activity is too high cells adopt a hindgut fate, but if either β-catenin or JNK activity is too low, as in Fzd7-MOs, foregut development is arrested (Zhang et al., 2013). Thus the Sdc4-MO phenotype could be due to disrupted JNK and/or β-catenin activity. Another possibility was that the disrupted foregut development caused by Sdc4 depletion was due to compromised BMP/Smad signaling. Ventral foregut progenitors require BMP signals from the cardiac mesoderm and the Fn1 matrix, which is absent in Sdc4-MOs, is essential to facilitate robust BMP signaling in this context (Kenny et al., 2012).

We assessed the status of β-catenin, JNK and Smad1 activation in Sdc4 depleted foregut tissue with the following transcriptional reporters plasmids: (1) TOP:flash containing multiple Tcf DNA-binding sites driving luciferase expression to measure canonical β-catenin/Tcf activity; (2) ATF2:luc containing multiple DNA-binding sites for the AP1 (Jun/Fos) transcription factor, which is phosphorylated and activated by JNK; and (3) BRE:luc with multiple Smad1-responsive elements driving luciferase. Each reporter was injected into presumptive foregut cells along with either control-MO, Sdc4-MOs or Fzd7-MOs and luciferase activity was measured at stage NF19. Sdc4 depletion resulted in reduced ATF2:luc and BRE:luc activity, which was rescued by co-injection of *sdc4* mRNA (Fig. 5A). In contrast Sdc4-depleted embryos and controls did not show a significant difference in TOP:flash activity. As expected both the β-catenin and JNK dependent reporters were reduced in Fzd7 depleted foregut tissue (Fig. 5A, Zhang et al., 2013). We also noted that Fzd7-MOs embryos exhibited a modest reduction in the BRE:luc activity, but not as dramatic as observed with the Sdc4-MOs (Fig. 5A). These data suggest that Sdc4 depletion results in impaired Wnt/JNK and BMP/Smad1 activity in the foregut, but no change in Wnt/β-catenin signaling.

As an independent method to validate the transcriptional reporters we performed confocal immunostaining for BMP and Wnt pathway effectors namely; phosphorylated Smad1/5/8 (pSmad1/5/8), phosphorylated JNK (pJNK) and nuclear β-catenin. In addition, to better understand the role of the Fn1 matrix we also compared Sdc4-depleted embryos with foregut specific depletion of Fn1 itself, or Szl, a Tolloid-protease inhibitor that is required for Fn1 matrix assembly and BMP signaling in the *Xenopus* foregut (Kenny et al., 2012). Using Image-J we quantified the average nuclear staining intensity of pSmad1/5/8, pJNK, and β-catenin in the foregut cells of Control MO, Sdc4-MO, Fzd7-MO, Fn1-MO and Szl-MO injected embryos.

Nuclear pSmad1/5/8 levels were significantly reduced in the foregut nuclei of Sdc4-MO, Fn1-MO and Szl-MO embryos compared to controls (Fig. 5B), which correlates with loss of the Fn1 matrix and impaired BMP signaling. Consistent with the BRE:luc data we also observed a modest, but not significant, reduction of pSmad1/5/8 in Fzd7-MO embryos, suggesting possible crosstalk between Wnt/Fzd7 and BMP pathways independent of the Fn1 matrix. Similar to the ATF2:luc assays, both Sdc4-MO and Fzd7-MO injected foreguts exhibited reduced nuclear pJNK. Fn1-MO injected cells also trended to have reduced pJNK, but this was variable and not statistically significant (Fig. 5C). Foregut depletion of Szl, which is known to cause a loss of Fn1 matrix assembly but does not impact overall Fn1 levels (Kenny et al., 2012), did not affect pJNK activity (Fig. 5C). Thus, unlike pSmad1, pJNK levels were not correlated with the presence or absence of the Fn1 matrix. This also suggested that in the *Xenopus* foregut, BMP signaling is unlikely to regulate JNK as described in some contexts (Grijelmo et al., 2007). Consistent with this conclusion pharmacological inhibition of BMP receptor activity resulted in a loss of pSmad1/5/8 and *hhex* in the foregut but had no impact on pJNK levels (Supplemental Fig. S4). Finally depletion of Sdc4, Fn1 or Szl had no impact on nuclear β-catenin levels, which as expected were down-regulated in Fzd7 depleted foreguts (Fig. 5D).

Another possible explanation for the reduced pJNK in Sdc4-MO embryos was reduced FGF signaling. FGFR activation can stimulate JNK (Ahn et al., 2009; Kim et al., 2014) and Sdc4 is known to modulate FGF in some contexts (Kuriyama and Mayor, 2009). To investigated this we treated embryos with FGFR inhibitors SU5402 or PD173074 (Shifley et al., 2012). Both inhibitors repressed cardiac mesoderm marker *nkx2.5*, a known FGF-target, but neither caused an obvious change in foregut *hhex* or pJNK levels (Supplemental Fig. S5A). Moreover phospho-FGFR and phosphor-ERK, two readouts of FGF signaling, showed no difference between control and Sdc4-MO foregut cells (Supplemental Fig. S5B). Together these results suggest that Sdc4 regulates JNK in the early foregut through non-canonical Wnt and not FGF.

We conclude that Sdc4 is required for robust Wnt/JNK and BMP/Smad1 signaling in the foregut. Unlike Fzd7, which transduces both JNK and β-catenin, signaling we observed that Sdc4 selectively promotes JNK activity. Moreover since β-catenin activity was not elevated in Sdc4-MOs we conclude that in the foregut Sdc4 does not inhibit canonical Wnt signaling as suggested in other contexts (Grumolato et al., 2010; Topol et al., 2003). Together with previous reports, our results suggest that Sdc4 promotes JNK activity possibly by forming a co-receptor complex with Fzd7. On the other hand our data suggest that Sdc4 facilitates pSmad1/5/8 activity by promoting assembly of the Fn1 matrix, which is required for robust BMP signaling.

3.6. Sdc4-Fn1 facilitates a positive BMP signaling loop maintaining bmp ligand expression

Previous studies indicate that in the Xenopus foregut BMP signaling forms a Fn1-dependent positive feedback loop, where Smad1 activity maintains robust *bmp* ligand expression and *hhex*+ foregut identity (Kenny et al., 2012). Consistent with the hypothesis that Sdc4 is required for this feedback loop we found that *bmp2, bmp4*, and *bmp7* transcripts in the foregut mesendoderm were reduced in Sdc4-MO, Fn1-MO and Szl-MO injected embryos (Supplemental Fig. S6). In contrast, *bmp2/4/7* expression was unaffected in Fzd7-depleted embryos, even though *hhex* was reduced. Expression of *wnt11* the mostly likely Fzd7 ligand in the foregut (Li et al., 2008), was unchanged by depletion of Sdc4, Fzd7, Fn1 or Szl (Supplemental Fig. S6). We conclude that Sdc4 is essential for the Fn1-mediated BMP feedback loop, which is independent of Fzd7/JNK activity. Moreover, the modest reduction in pSmad1/5/8 observed in Fzd7-depleted embryos (Fig. 5) appears insufficient to cause a collapse of the BMP signaling loop that maintains *bmp* ligand expression.

3.7. BMP regulates foregut identity whereas Wnt/JNK regulates both foregut identity and morphology

To determine whether different aspects of the Sdc4 loss-of-function phenotype were due to reduced BMP/Smad1 or Wnt/JNK signaling we compared the ability of BMP2 protein or mRNA encoding constitutively active JNK (caJNK), both injected into the foregut, to rescue the cell morphology or *hhex* expression in Sdc4-depleted embryos. Immunostaining confirmed that injection of recombinant BMP2 protein to the Sdc4-MO foregut restored pSmad1/5/8 levels but not pJNK levels, whereas caJNK had no effect on pSmad1/5/8 (Fig. 6; Supplemental Fig. S7). Injection of BMP2 protein was unable to rescue the cell morphology defects in the Sdc4-MO foreguts (Fig. 6N), whereas the caJNK restored cortical

β-catenin and foregut cell morphology (Fig. 6M). In contrast BMP2 protein or caJNK both partially rescued *hhex* expression (Fig. 6R and S), indicating that BMP signaling and JNK activity both contribute to foregut specific gene expression.

Injection of BMP2 protein was also sufficient to rescue *hhex* and *bmp4/7* expression in Szl-MO and Fn1-MO injected embryos (Supplemental Fig. S8), suggesting that the Fn1 matrix is not absolutely required for BMP signaling in the presence of excess exogenous ligand. In contrast BMP2 protein injection did not restore *hhex* expression in Fzd7-depleted embryos (Supplemental Fig. S8) demonstrating that even in an environment with excess BMP foregut fate requires a low level of Wnt/Fzd signaling. We conclude that Sdc4 is required for both Wnt/JNK and BMP/Smad1, which are essential to maintain ventral foregut progenitors.

3.8. The Sdc4 cytoplasmic PDZ binding motif is dispensable for Fn1-dependant BMP signaling, but required for foregut cell morphology

Sdc4 protein has three major domains: an extracellular domain that interacts with Fn1, a transmembrane domain and an intracellular domain containing a highly conserved PDZ binding motif (PBM), which physically binds the Wnt/Fzd-effector protein Disheveled (Carvallo et al., 2010).

We hypothesized that the intracellular PBM might be critical for non-canonical Fzd/JNK signaling whereas the other portions of Sdc4 including extracellular domain might be sufficient for the assembly of Fn1 matrix and BMP signaling in the foregut. To test this, we injected mRNA encoding wild-type Sdc4 or a truncated form of Sdc4 that lacks the intracellular PBM (Sdc4ΔPBM) (Carvallo et al., 2010) into D1 presumptive foregut cells after Sdc4-MO injection. The Fn1 matrix between foregut mesoderm and endoderm as well as nuclear pSmad1/5/8 immunostaining was restored by either wild type Sdc4 or Sdc4ΔPBM (Fig. 7A–H). On the other hand, only the wild type Sdc4 but not the Sdc4ΔPBM rescued the foregut cell morphology in Sdc4-depleted embryos (Fig. 7I and K). Expression of *hhex* was only partially rescued by Sdc4ΔPBM (Fig. 7M and O), which is consistent with the observation that exogenous BMP ligand also only partially rescued *hhex* in Sdc4-depleted embryos.

We conclude that the intracellular PBM domain of Sdc4 containing is necessary for JNK-mediated foregut cell morphology and robust gene expression, whereas other portion of Sdc4 including the ectodomain are sufficient to support the Fn1 matrix assembly and BMP signaling in the foregut. Thus Sdc4 coordinates foregut progenitor development in *Xenopus* by coordinating Wnt and BMP activity in the extracellular space.

4. Discussion

Wnt and BMP are critical for foregut progenitor maintenance but how their activities are coordinated is poorly understood. We show that the extracellular HSPG Sdc4 modulates both Fzd7/JNK and BMP/Smad1 signaling to orchestrate *Xenopus* foregut progenitor development. We propose a model where Sdc4 coordinately regulates BMP and Wnt activity *via* two different mechanisms (Fig. 8). First, our data suggest that Sdc4 and Fzd7 co-operate to stimulate an intracellular JNK signal transduction cascade that is required to maintain

foregut gene expression, proliferation, and cell adhesion. Second, our data suggest that Sdc4 indirectly promotes BMP signaling by stimulating the formation of the Fn1 matrix in the foregut. This Fn1 matrix is required to maintain a positive BMP feedback loop that is essential for ventral foregut gene expression (Kenny et al., 2012).

Regarding the Sdc4/Fz7/JNK mechanism, previous studies have shown that Sdc4 and Fn1 can physically associate with Fzd7 to form a co-receptor complex that stimulates membrane recruitment of Disheveled and the activation of a non-canonical Wnt signaling cascade including JNK and small Rho GTPases (Bentzinger et al., 2013; Kraft et al., 2012; Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011). Our data support a similar co-receptor role for Sdc4 in the *Xenopus* foregut that specifically promotes non-canonical Wnt/JNK pathway. Epistasis and rescue data indicate that Sdc4 and Fzd7 together stimulate JNK activity in the foregut and that the intracellular PDZ-binding domain of Sdc4, which is known to interact with Disheveled, is required for this activity. Based on the transcriptional reporter assay it is likely that Sdc4/Fzd7-stimulated JNK activity regulates foregut gene expression and proliferation by the activation of Jun/Fos (AP1) transcription factors. Our data indicate that the Sdc4/Fzd7/JNK pathway also regulates cell adhesion and cell shape in the *Xenopus* foregut and that this occurs in part by stabilizing Cdh3 levels. This is consistent with reports that Wnt11/Fzd7 signaling can regulate endocytic Cadherin recycling to promote cell-cohesion in early gastrula cells (Dzamba et al., 2009; Kraft et al., 2012; Ohkawara et al., 2011; Ulrich et al., 2005). Fzd7 also regulates separation of the ectoderm and chordomesoderm at the involuting marginal zone of the gastrula *via* PKC signaling (Winklbauer et al., 2001) and it is possible that Sdc4 may also act as co-receptor in this context. Pharmacological inhibition of JNK results in a phenotype similar to what we observe here with depletion of either Sdc4 or Fzd7 (Zhang et al., 2013). JNK regulation of foregut cell adhesion and the cytoskeleton may be direct as JNK activity is required for the association of β-catenin and Cadherins in culture mammalian keratinocytes (Lee et al., 2006), and previous studies have shown that disruption of Catenin-Cadherin interactions can result in elevated proteasome degradation of Cadherins (Kowalczyk and Reynolds, 2004). It is likely that small GTPases are also involved as Sdc4-Fzd interactions can stimulate Rac1 to modulate the cytoskeleton during neural crest cell migration (Matthews et al., 2008).

Recent publications indicate that Sdc4 can also repress canonical Wnt function in some contexts (Astudillo et al., 2014), however we observed very little, if any impact on β-catenin signaling in the Sdc4-depleted foregut. Indeed our previous results indicate that Wnt11 signaling through Fzd7 stimulates a low level of both JNK and β-catenin activity in the *Xenopus* foregut (Li et al., 2008; Zhang et al., 2013). Thus Sdc4 and Lrp6 co-receptors probably control the relative level of non-canonical versus canonical signaling in the foregut respectively. Recent studies indicate that Rspo3 and Vangl2 proteins also interact with Sdc4-Fn1-Fzd complexes to promote Wnt/PCP signaling in some contexts (Escobedo et al., 2013; Li et al., 2008; Ohkawara et al., 2011; Zhang et al., 2013) and it will be interesting to examine the role of these in foregut progenitor development.

Regarding the Sdc4/-regulated BMP/Smad1 activity (Fig. 8), our data suggest that Sdc4 facilitates BMP signaling by promoting the assembly of Fn1-rich ECM. Fn1 matrices are assembled when soluble Fn1 dimers bind to Sdc4 and Integrin α5β1 complexes on the cell

surface, and then with the help of cadherin-mediated cell tension, Fn1 dimer undergoes a conformational change causing self-assembly into fibrils (Dzamba et al., 2009; Ramirez and Rifkin, 2009; Rozario et al., 2009; Schwarzbauer and DeSimone, 2011). Consistent with previous analyses of Fn1-MO and Szl-MO injected embryos (Kenny et al., 2012), loss of the Fn1 matrix between the BMP-producing mesoderm and the foregut endoderm in Sdc4-depleted foregut tissue correlates with reduced pSmad1 activity and a collapse of the positive BMP signaling loop including a failure to maintain *bmp2/4/7* and *hhex* transcripts. The fact that the Sdc4 protein lacking intracellular PDZ-binding domain is sufficient to support Fn1 matrix and pSmad1 activity suggests that Sdc4-Disheveled signaling is not required to promote BMP signaling in the foregut. Sdc1 and Sdc3 have also been reported to modulate BMP signaling (Fisher et al., 2006; Olivares et al., 2009), but the mechanisms are unknown and it remains to be determined if the Fn1 matrix is involved. Precisely how the Fn1 matrix promotes BMP signaling is currently unclear. Fn1 is a pioneering ECM protein, it is one of the first matrix proteins laid down and recruits other HSPGs and ECM proteins (Schwarzbauer and DeSimone, 2011), including Fibrillin, Collagen IV and Biglycan, all of which have been implicated to some extent in BMP-binding through heparan sulphate interaction (Hacker et al., 2005; Lin, 2004). Our working hypothesis is that Sdc4, Fn1 and/or associated HSPGs help increase the local concentration of BMP ligands and may facilitate receptor BMP-interactions.

Interestingly Sdc4 is also implicated in *Xenopus* neural induction by regulating FGF signaling (Kuriyama and Mayor, 2009). Although our data suggest that Sdc4 does not regulate Sdc4-dependent FGF signaling in the early foregut progenitors Sdc4-FGF may act later in foregut organogenesis. We have previously shown that prolonged FGF activity from NF19–35 is required for liver development and the late liver hypoplasia we see in Sdc4-MOs at stage NF35 could be due in part to compromised FGF singaling.

In mouse, Sdc4 is expressed in the foregut progenitors at E8.5 and E9.0, similar to *Xenopus*, however *Sdc4*−/− germ-line null mice are viable with defects in wound healing and angiogenesis (Echtermeyer et al., 2001). It is thought that other Syndecan family members, all of which broadly expressed, can probably compensate for one another in mammals (Alexopoulou et al., 2007; Echtermeyer et al., 2001; Escobedo et al., 2013) It is also possible that in mammals other ECM enriched HSPG such as Glypican or Collagen may be able to mediate ECM regulation of Wnt and/or Bmp signaling in the absence of Sdc4. In the future it will be important to examine the status of Wnt and BMP signaling in Syndecan mutant mice. For example there is evidence that both non-canonical Wnt signaling and BMP signaling plays a role in would healing in mammals (Fathke et al., 2006; Lyu and Joo, 2005; Werner and Grose, 2003), raising the possibility that the defective would healing in *Sdc4*−/− mutant mice may be due in part to disrupted Wnt and/or BMP activity. Thus the mechanism by which Sdc4 and Fn1 coordinate Wnt and BMP signaling that we describe here may have broad implications for other development and disease contexts.

5. Conclusions

Using a foregut specific loss-of-function analysis, we demonstrated that Sdc4 is required for Fzd7-dependent non-canonical Wnt signaling and Fn1-dependent BMP signaling to

coordinate cell fate, morphogenesis and proliferation during *Xenopus* foregut organogenesis. This exemplifies how an HSPG and modulation of the ECM can integrate the activity of different signaling pathways in development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.05.025.

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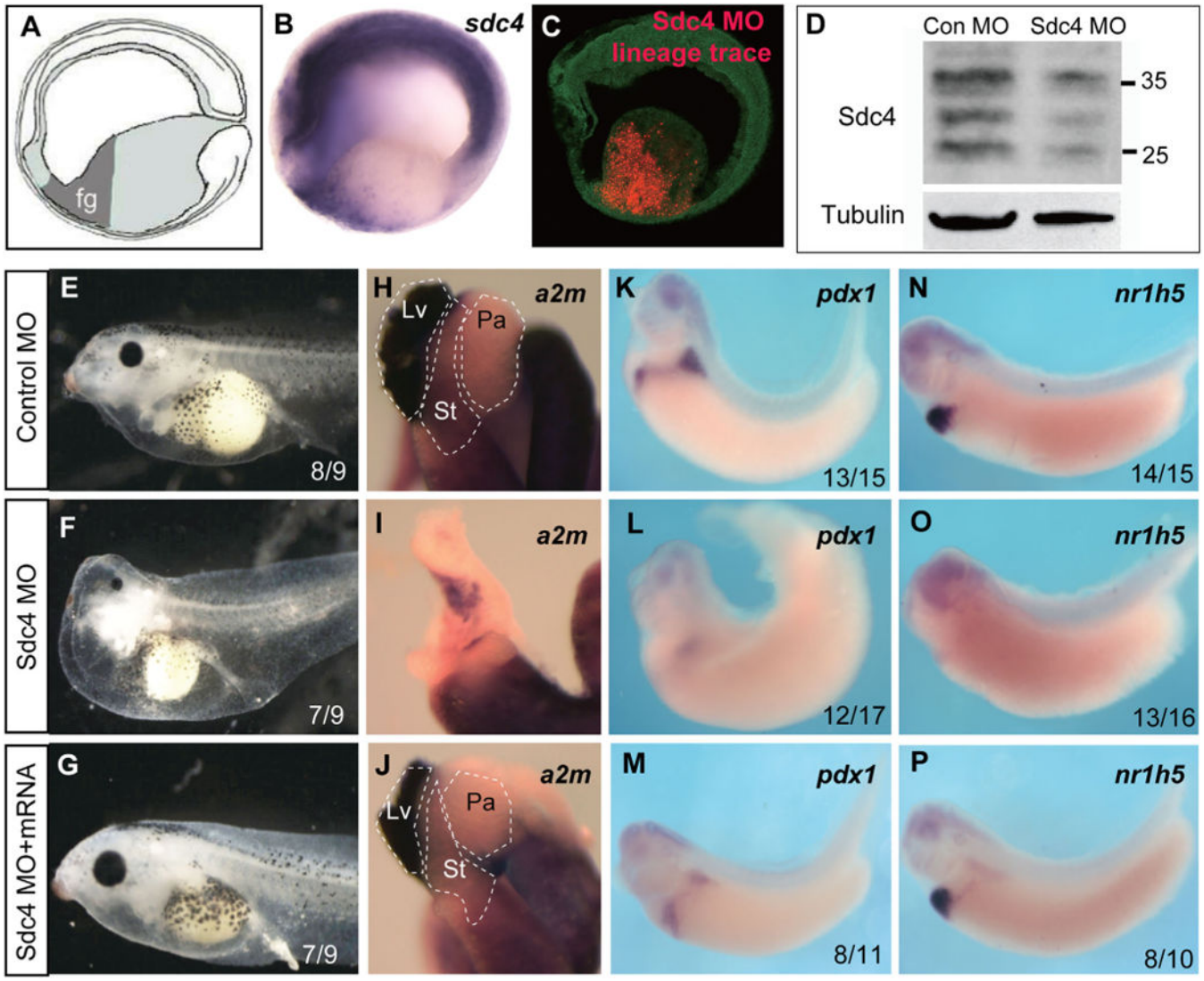
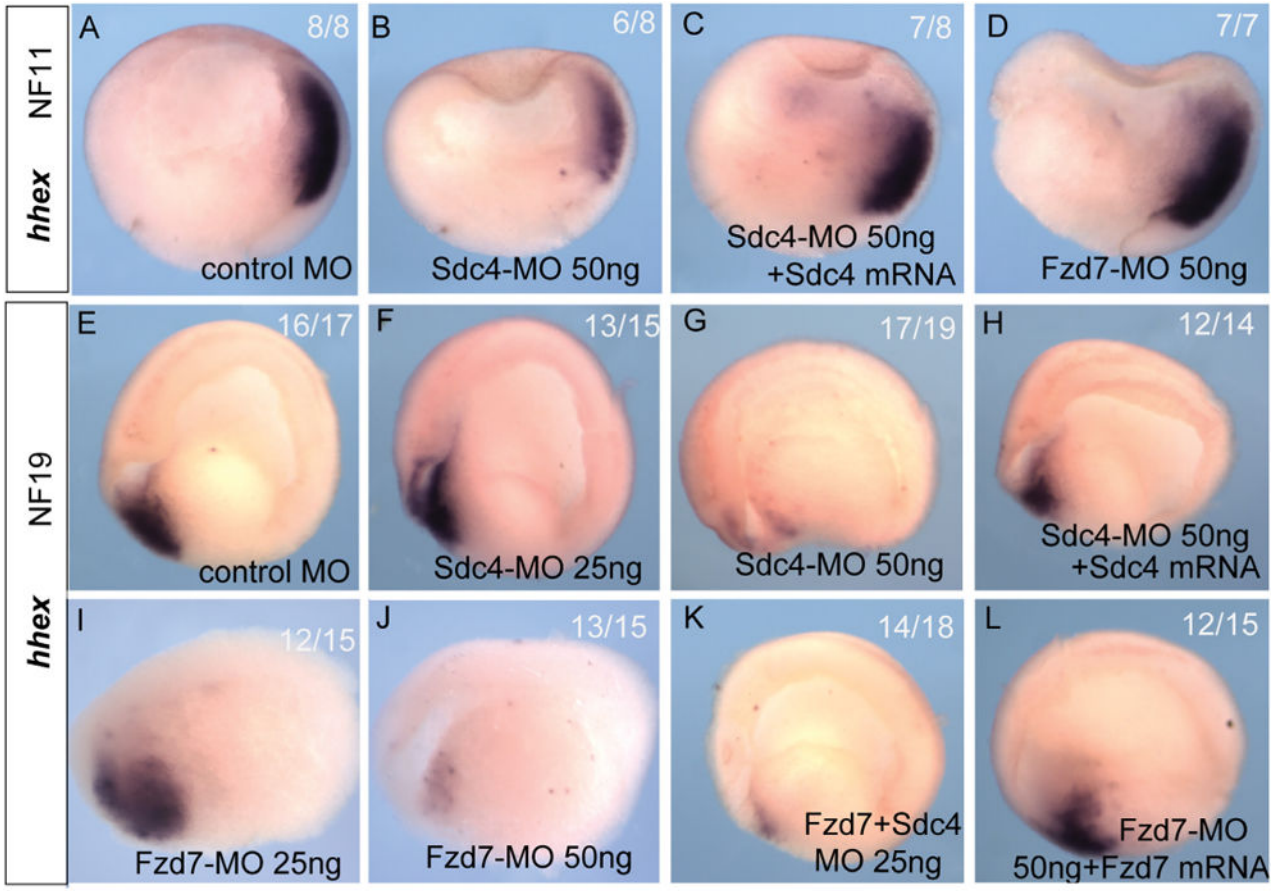


Fig. 1.

Sdc4 is required for foregut organogenesis (A) Schematic of a NF19 stage *Xenopus* embryo in mid-sagittal section with the foregut (fg) endoderm shown in dark grey and mid/hindgut endoderm in light grey. (B) *In situ* hybridization showing *sdc4* expression at stage NF19. (C) Confocal image showing Alexa lineage tracer (red) in a bisected stage NF19 embryo confirming the targeted microinjection of the foregut endoderm. (D) Western blot analysis of NF19 dissected foreguts confirming Sdc4 depletion by Sdc4-MO injection. (E–G) Lateral view of stage 45 embryos showing foregut hypoplasia in Sdc4-depleted embryos that is rescued by *sdc4* mRNA injection. (H–J) *In situ* hybridization of *a2m* in isolated stage 45 gut tube showing hypoplastic liver (lv), stomach (st) and pancreas (p). (K–P) *In situ* hybridization with liver (*nr1h5*) and pancreas/duodenum (*pdx1*) markers at stage NF35 showing defects in organ specification, which are rescued by *sdc4* RNA injection. In all panels, numbers indicate the embryos exhibiting the phenotype.



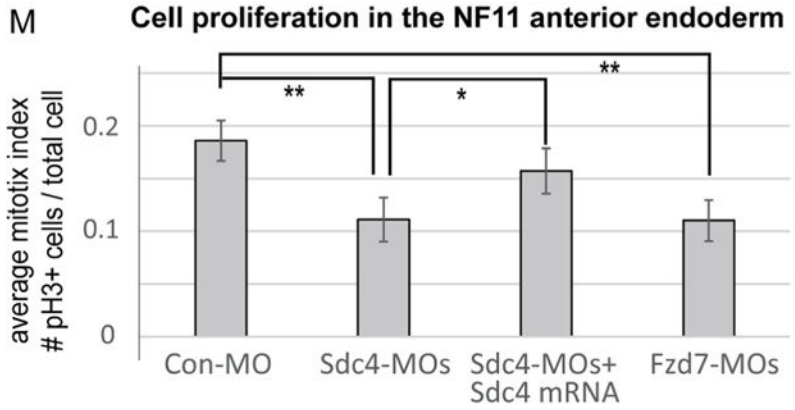


Fig. 2.

Sdc4 is required for early foregut progenitor gene expression and proliferation (A–L) *In situ* hybridization with foregut progenitor marker *hhex* on embryos injected with indicated dose of control-MO, Sdc4-MO and/or Fzd7-MO at stage NF11 (A–D) and stage NF19 (E–L). (M) Loss of Sdc4 and Fzd7 results in reduced anterior endoderm proliferation at NF11. The mitotic index (pH3+ cells/total foregut cells) was quantified from confocal phosphor-histone H3 immunostaining of bisected NF11 embryos injected with the indicated MO and mRNAs.

The average mitotic index ± S.D. *p<0.05 and**p<0.01 relative to age matched controls in Student's *t*-test, (n=4 embryos/condition with 100 cells/embryo).

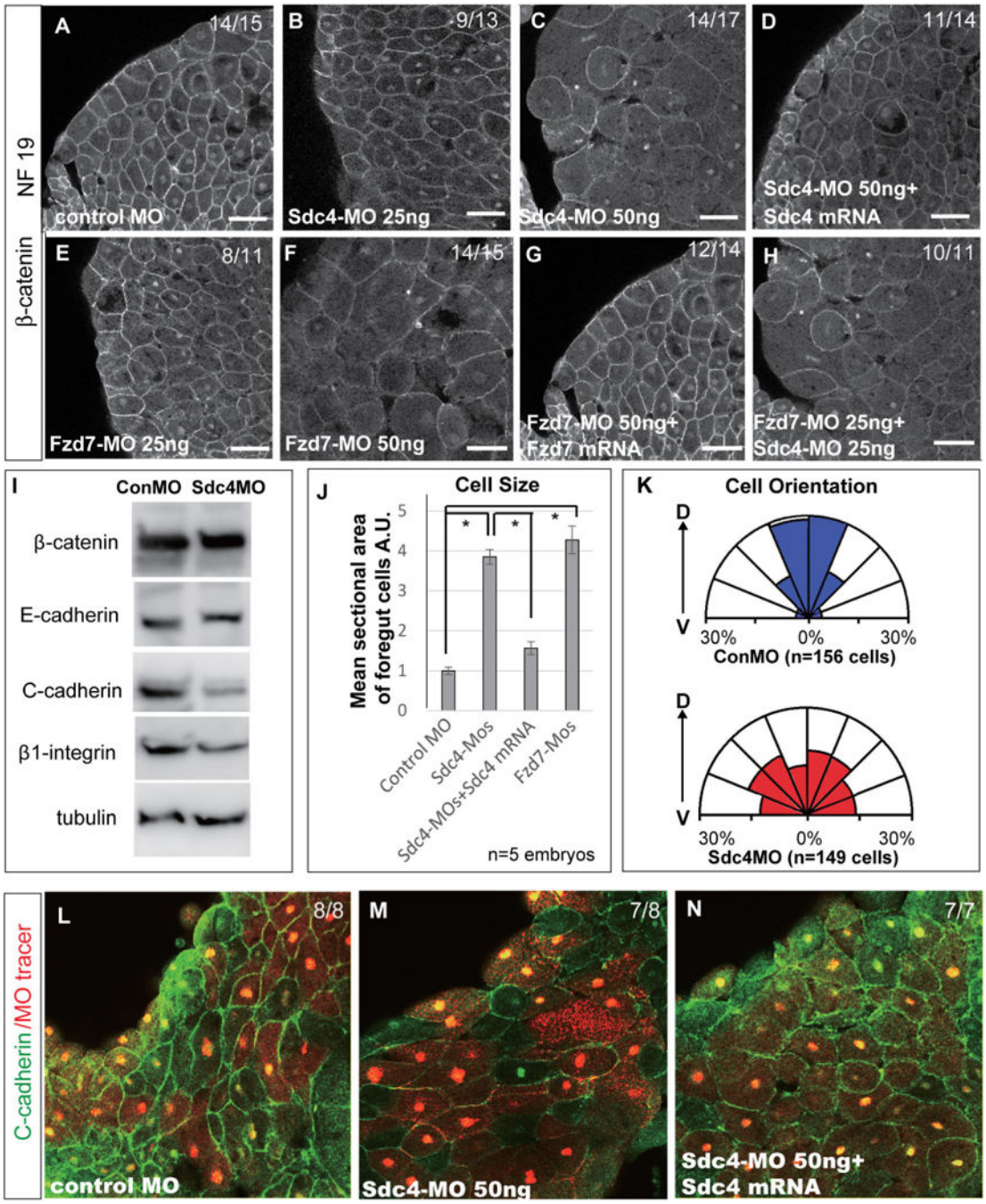


Fig. 3.

Sdc4 interacts with Fzd7 to regulate cell adhesion and morphology of foregut progenitors (A–H) Confocal immunostaining of cytoskeletal β–catenin in the foregut of NF19 bisected embryos injected with the indicated MO and/or mRNAs. (I) Western blot analyses of stage NF19 dissected foregut tissue shows no obvious changes of total β-catenin, E-cadherin and Tubulin levels, whereas Cdh3 and Itgb1 levels are reduced by 71% and 48%, respectively (quantified by Image-J). (J) Quantitation of foregut cell size in control MO, Sdc4-MO and Fzd7-MO injected embryos were measured from β–catenin immunostaining using Image-J.

*p<0.05 in pairwise student *T*-tests. K) Quantification of foregut cell orientation (direction of the long axis of the cell relative to the Dorsal (D)–Ventral (V) axis) in control and Sdc4-depleted embryos (4 embryos of each condition). (L–N) Confocal immunostaining of the anterior endoderm at stage NF11 showing Cdh3 (C-cadherin) in green and Sdc4-MO/RLDx in red.

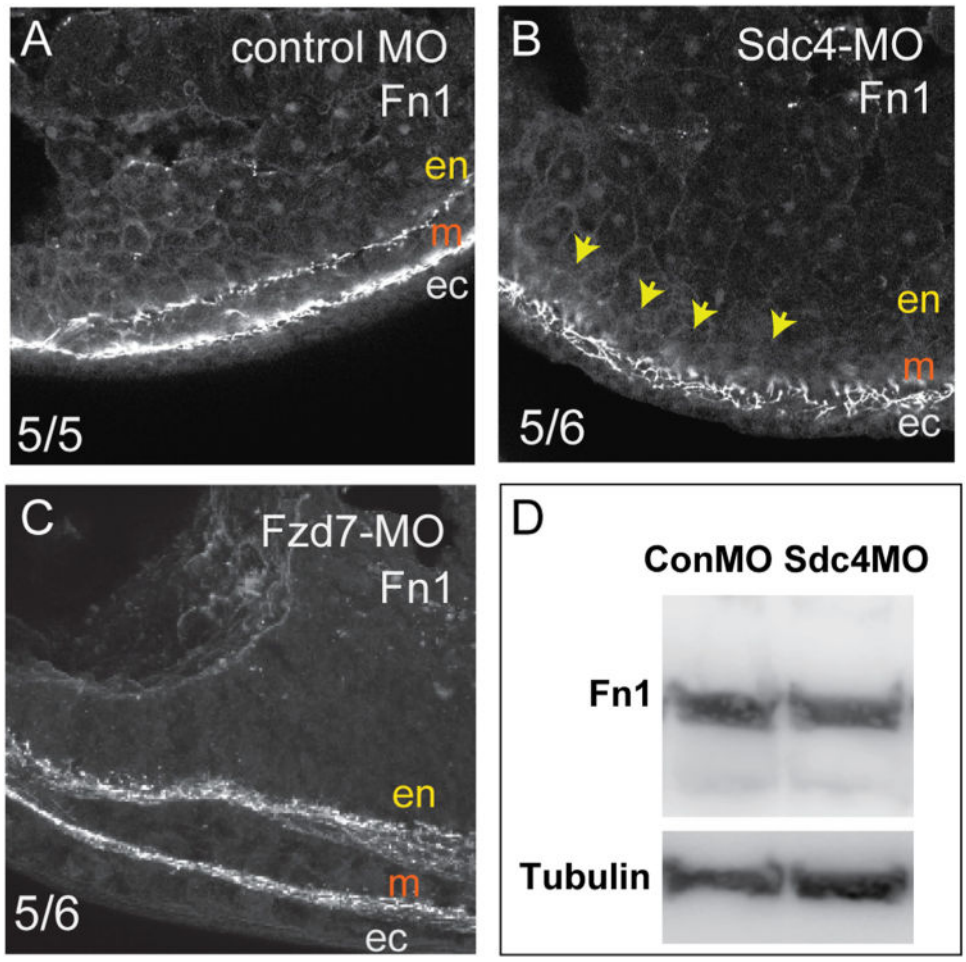


Fig. 4.

Sdc4 mediates Fn1 matrix deposition in the foregut (A–C) Confocal Fn1 immunostaining in the foregut of control-MO, Sdc4-MO and Fzd7-MO injected embryos at stage NF19. In controls peri-cellular Fn1 is observed in the foregut around the closing blastocoel and two Fn1 matrix layers: one between the foregut endoderm (en) and cardiac mesoderm, (m) and the other layer between foregut mesoderm (m) and ectoderm (ec). The Fn1 layer between the endoderm and mesoderm is largely absent (arrows) in Sdc4-depleted embryos. (D) Western blot analysis of dissected NF19 foreguts tissue shows that total Fn1 protein levels are unchanged in Sdc4-depleted embryos as compared to controls.

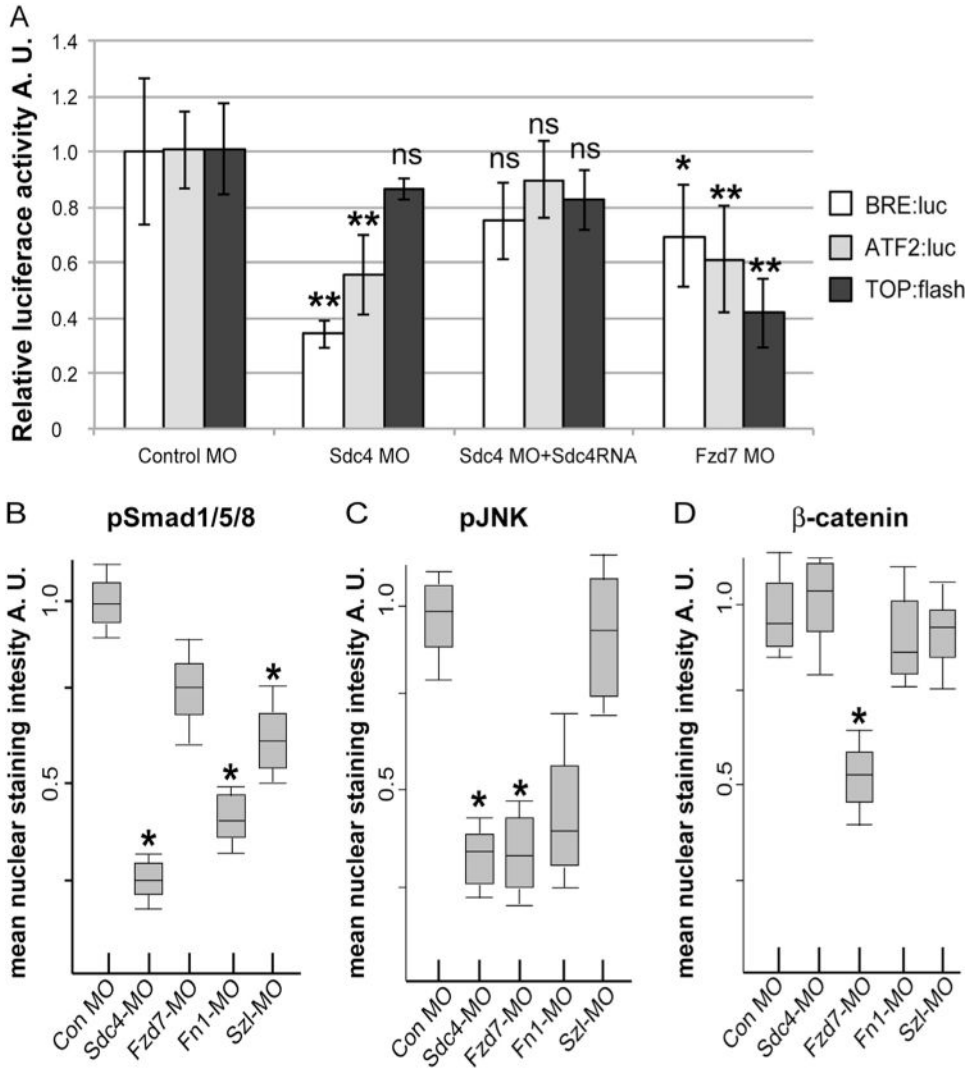


Fig. 5.

Sdc4 is required for both Wnt/JNK and BMP/Smad signaling in the foregut (A) Luciferase reporter assays in control-MO, Sdc4-MO, and Fz7-MO injected embryos. Firefly luciferase reporter plasmids (BRE for BMP activity, ATF2 for Wnt/JNK activity, and TOP:flash for canonical Wnt/β – catenin activity) were co-injected with pRL-TK: Renilla luciferase into D1 presumptive foregut cells of 32-cell stage embryos and assayed at stage NF19. Luciferase activity was normalized to renilla activity and the mean relative activity of triplicate samples is shown ± S.D; injections were repeated a minimum of three times and a representative result is shown. *p<0.05 and **p<0.01 in pairwise student *t*-tests compared to control-MO reporter levels. (B–D) Quantification of confocal immunostaining of pSmad1/5/8, pJNK and nuclear β – catenin. Mean pixel intensity of normalized to nuclear sytox green measured using Image-J. Arbitrary units (A.U.) with control-MO samples set to 1.0±S.D. *p<0.05 in repeated measure ANOVA (n=5 embryos/condition with 50–150 cells/embryo).

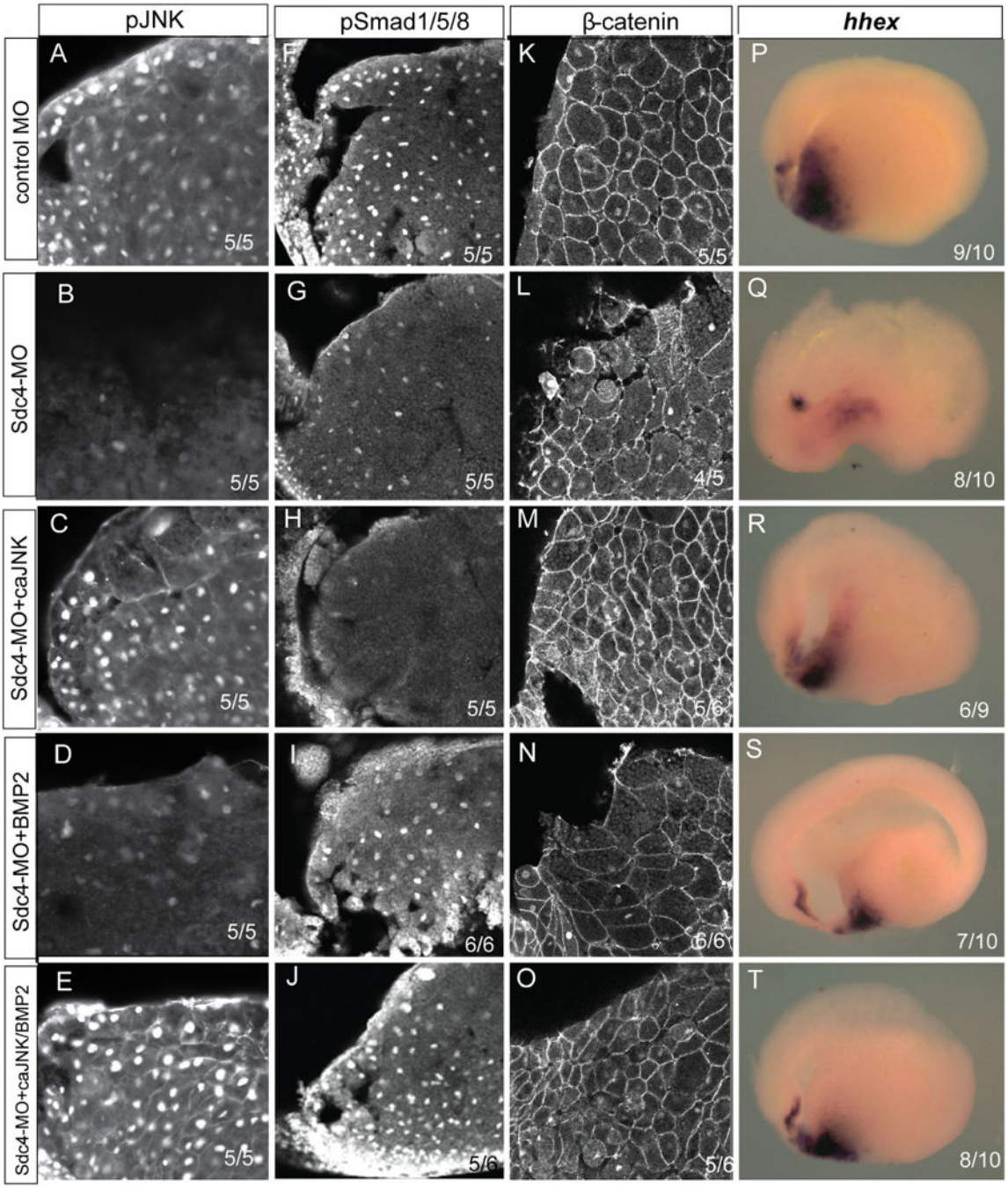


Fig. 6.

BMP signaling regulates foregut identity while Wnt/JNK signaling is necessary for both foregut identity and morphology (A–E) Confocal immunostaining of phosphorylated JNK (pJNK) or (F–J) phosphorylated Smad1/5/8 (pSmad1) in the foregut of bisected embryos at stage NF19. Quantification showed in Supplemental Fig. S7 (K–O) Confocal immunostaining of cortical β-catenin as cell membrane marker to show foregut cell morphology at NF19. (P–T) *In situ* hybridization with *hhex* in bisected NF19 embryos. The

number of embryos with representative phenotype on the left versus total embryos examined on the right.

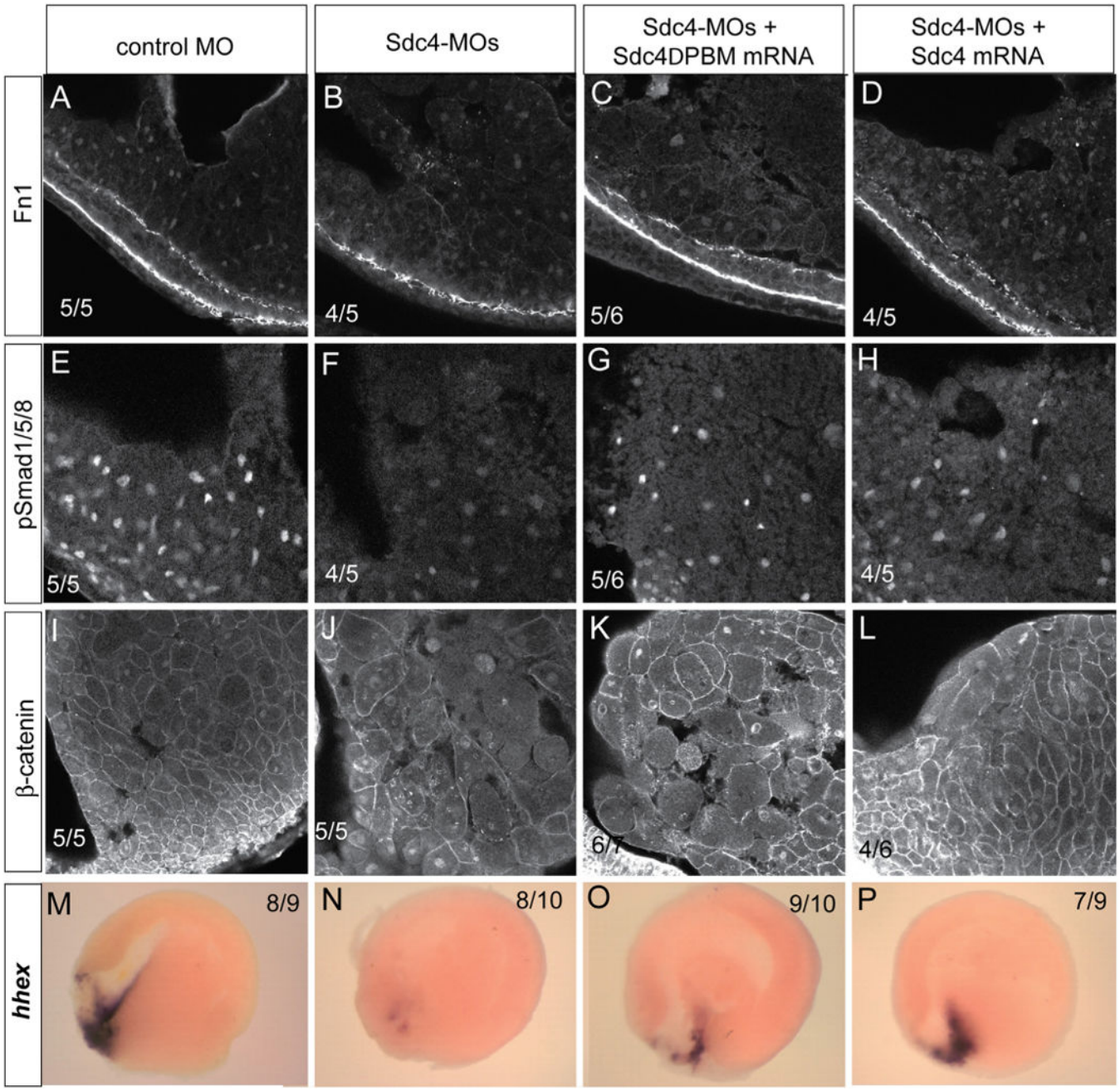


Fig. 7.

The Sdc4 PDZ-binding domain is dispensable for Fn1 matrix assembly and BMP signaling, but required cell morphology (A–D) Confocal Fn1 immunostaining in the foregut region of bisected embryos at stage NF19. (E–H) Confocal immunostaining of pSmad1/5/8 at NF19. Quantification showed in Supplemental Fig. S7 (I–L) Confocal immunostaining of cytoskeletal β-catenin showing foregut cell morphology at NF19. (M–P) *In situ* hybridization with *hhex*. Numbers in the figure show the number of embryos with representative phenotype on the left versus total embryos examined on the right.

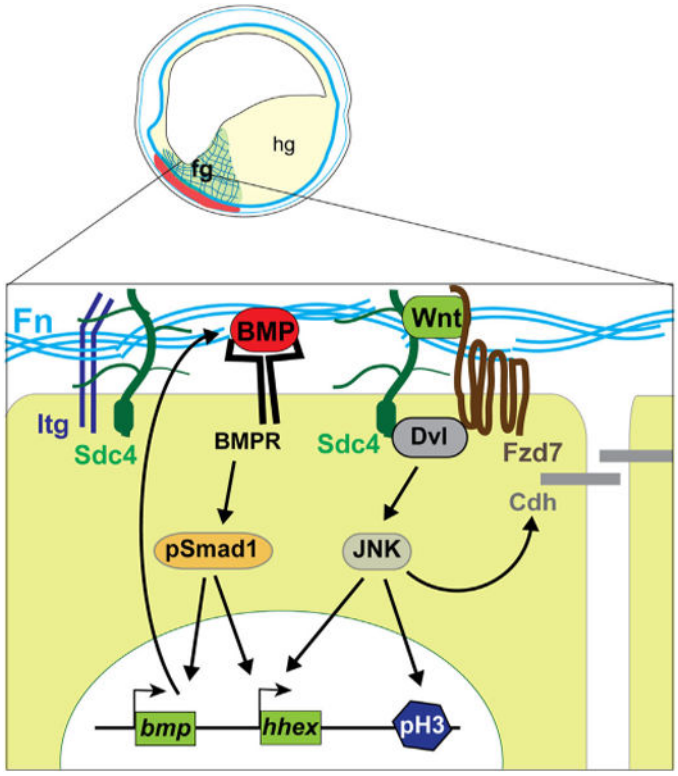


Fig. 8.

A model suggesting how Sdc4 might coordinate Fn1-mediated BMP signaling and Fzd7-mediated Wnt/JNK signaling in the foregut The schematic on top shows a section through a Xenopus embryo with a peri-cellular Fn1 matrix (blue) present throughout the foregut (fg) and a prominent Fn1 layer between the endoderm and the BMP-expressing cardiac mesoderm (red). The blowup shows a foregut progenitor cell with integrin (Itg) and Sdc4 complexes promoting Fn1 matrix assembly (blue). We postulate that the Fn1 matrix might enhance BMP-receptor interactions to stimulate Smad1/5/8 phosphorylation (pSmad1), which are required to maintain a positive BMP feedback loop and *hhex* expression. In this model Sdc4-Fn1 also promotes non-canonical Wnt signaling by forming a co-receptor complex with Fzd7 to recruits Dishevelld (Dvl) and activate JNK. Activation of JNK would then promote *hhex* expression, stimulate foregut cell proliferation (pH3) and stabilize Cdh on the foregut cell surface.