

# Repression of Wnt/ $\beta$ -catenin signaling in the anterior endoderm is essential for liver and pancreas development

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The liver and pancreas are specified from the foregut endoderm through an interaction with the adjacent mesoderm. However, the earlier molecular mechanisms that establish the foregut precursors are largely unknown. In this study, we have identified a molecular pathway linking gastrula-stage endoderm patterning to organ specification. We show that in gastrula and early-somite stage *Xenopus* embryos, Wnt/ $\beta$ -catenin activity must be repressed in the anterior endoderm to maintain foregut identity and to allow liver and pancreas development. By contrast, high  $\beta$ -catenin activity in the posterior endoderm inhibits foregut fate while promoting intestinal development. Experimentally repressing  $\beta$ -catenin activity in the posterior endoderm was sufficient to induce ectopic organ buds that express early liver and pancreas markers.  $\beta$ -catenin acts in part by inhibiting expression of the homeobox gene *hhx*, which is one of the earliest foregut markers and is essential for liver and pancreas development. Promoter analysis indicates that  $\beta$ -catenin represses *hhx* transcription indirectly via the homeodomain repressor Vent2. Later in development,  $\beta$ -catenin activity has the opposite effect and enhances liver development. These results illustrate that turning Wnt signaling off and on in the correct temporal sequence is essential for organ formation, a finding that might directly impact efforts to differentiate liver and pancreas tissue from stem cells.

**KEY WORDS:** Endoderm, Patterning, Liver, Pancreas, Foregut, Wnt,  $\beta$ -catenin, Wnt-antagonists, Gsk3 $\beta$ , *Xenopus*, *hhx*, *foxa2*, *vent2*

## INTRODUCTION

The liver and pancreas are derived from the foregut endoderm. Although we increasingly understand the genetic pathways regulating proliferation and differentiation of these organs once they are specified (McLin and Zorn, 2006; Zaret, 2002; Zhao and Duncan, 2005), the earlier events linking endoderm formation at gastrulation to organ induction are less clear. A detailed understanding of these early developmental decisions is likely to be important for designing efficient strategies to generate therapeutically useful tissue from stem cells.

Hepatic specification in the ventral foregut endoderm requires signals from the surrounding mesenchyme (Fukuda-Taira, 1981; Gualdi et al., 1996; Le Douarin, 1975). In the mouse and chick, these signals include Fgf2 from the cardiogenic mesoderm and BMPs from the septum transversum mesenchyme (Jung et al., 1999; Rossi et al., 2001; Zhang et al., 2004), whereas in zebrafish Wnt2bb in the lateral plate mesoderm is required (Ober et al., 2006). There is also evidence that prior to these signals, there are earlier developmental decisions that are crucial for the hepatic lineage. For example, transplantation and explant studies in amphibian and chick embryos have shown that only the foregut endoderm, but not the posterior endoderm, is competent to become liver in response to signals from the cardiogenic mesoderm (Fukuda-Taira, 1981; Le Douarin, 1975; Okada, 1954b; Okada, 1960; Takata, 1960). In addition, tissue recombination experiments have shown that an unknown signal from the trunk mesoderm represses hepatic potential in the posterior

endoderm (Gualdi et al., 1996; Le Douarin, 1975; Takata, 1960). Together, these findings suggest that establishment of the foregut domain prior to hepatic induction is an essential prerequisite for liver development.

The regional expression of marker genes along the anterior-posterior (A-P) axis of the gut tube demonstrates that distinct foregut, midgut and hindgut domains exist prior to organ specification (Costa et al., 2003; Gamer and Wright, 1995; Grapin-Botton, 2005; Newman et al., 1997). However, studies on the mechanisms controlling this have been somewhat contradictory. Some experiments in amphibians suggested that regional identity in the endoderm is determined by gastrulation (Hamburger, 1996; Holtfreter, 1938), whereas other studies indicated that continual interactions with the mesoderm are required (Okada, 1954a; Okada, 1954b; Okada, 1960; Takata, 1960). For example, anterior vegetal tissue isolated from *Xenopus* blastulae will autonomously express foregut genes owing to intrinsic Nodal and Wnt signaling in the explants (Gamer and Wright, 1995; Henry et al., 1996; Zorn et al., 1999). However Horb and Slack subsequently detected cardiac and lateral mesoderm markers in such vegetal explants (Horb and Slack, 2001), suggesting that prolonged mesodermal interactions might be required to maintain regional identity, although the molecular details of this remain poorly understood.

In this study we postulated that differential zygotic Wnt signaling might regulate endoderm patterning similar to the 'activation-transformation' model of A-P patterning in the neural tube. In this model, neural tissue is initially anterior in character and is then progressively posteriorized by Wnts expressed in the trunk mesoderm, whereas anterior identity is maintained rostrally where there is a lack of Wnt signaling (Christian and Moon, 1993; Erter et al., 2001; Kiecker and Niehrs, 2001; Kim et al., 2000; Lekven et al., 2001; McGrew et al., 1995; Nieuwkoop, 1999; Takada et al., 1994). In the canonical pathway, Wnt ligands interact with a Frizzled-LRP5/6 receptor complex causing the inactivation of a Gsk3 $\beta$ -containing intracellular complex that would otherwise promote  $\beta$ -catenin degradation. This results in the accumulation of nuclear  $\beta$ -

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catenin, which interacts with Tcf/Lef transcription factors to activate target gene transcription (Clevers, 2006). Secreted Wnt-antagonists block signaling by binding to Wnt ligands in the extracellular space (sFRPs), or by binding to the LRP co-receptor (Dkk1) (Kawano and Kypta, 2003; Mao et al., 2001).

In *Xenopus*, a maternal Wnt pathway results in high levels of nuclear  $\beta$ -catenin in the dorsal-anterior endoderm of the blastula, which specifies the dorsal-anterior axis of all three germ layers (Heasman, 2006). However, during gastrula and early somite stages, nuclear  $\beta$ -catenin levels are rapidly reduced in the anterior endoderm relative to the posterior endoderm (Schohl and Fagotto, 2002). We postulated that this region of low  $\beta$ -catenin activity is important to maintain foregut identity and that Wnt ligands (Wnt8, Wnt8b, Wnt3, Wnt3a and Wnt1) expressed in the trunk mesoderm (Christian et al., 1991; Kemp et al., 2005; Moon, 1993) signal to the posterior endoderm to inhibit foregut/hepatic fates and promote intestinal development. By contrast, Wnt-antagonists (Dkk1, Frzb1, Crescent and Sfrp5) secreted by the anterior endoderm (Kemp et al., 2005; Leyns et al., 1997; Pilcher and Krieg, 2002; Wang et al., 1997) would protect it from the posteriorizing Wnt ligands, keep nuclear  $\beta$ -catenin levels low, and maintain foregut identity. Although such spatial interactions between zygotic Wnt ligands and their antagonists were known to regulate mesoderm and neural tube patterning, their roles in the early endoderm had not been established.

Consistent with this hypothesis, we find that forced Wnt/ $\beta$ -catenin signaling in the anterior endoderm, between gastrula and early somite stages, inhibits foregut development. By contrast, blocking  $\beta$ -catenin activity in the posterior endoderm is sufficient to initiate ectopic liver and pancreas development. This suggests that the endoderm is indeed patterned by differential Wnt signaling, similar to what has been described in the nervous system. The homeobox gene *hhex*, one of the earliest foregut markers, is a target of this  $\beta$ -catenin-mediated patterning and Hhex function is required for both normal and ectopic liver and pancreas development. Analysis of the *hhex* promoter indicates that  $\beta$ -catenin/Tcf activity represses *hhex* transcription in the posterior endoderm indirectly via homeodomain transcriptional repressor Vent2. This is a novel function for Vent factors, which are best known as mediators of BMP signaling in the mesoderm. These results provide a molecular pathway linking endoderm patterning to the initiation of liver and pancreas development, and illustrate that the spatial and temporal activity of Wnt signaling must be tightly controlled during this process.

## MATERIALS AND METHODS

### Embryo manipulations and microinjections

*Xenopus laevis* embryos were cultured as previously described (Zorn et al., 1999) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Explants were microdissected with tungsten needles and cultured in 0.5×MBS (Zorn et al., 1999) with antibiotics. For tissue separations, explants were cultured in 10  $\mu$ g/ml dispase for 15–20 minutes. Gastrula-stage endoderm tissue was transplanted into host embryos through an incision in the blastocoel roof, as previously described (Slack and Isaacs, 1994).

Embryos with clear pigmentation differences between dorsal-anterior and ventral-posterior cells were selected for 32-cell stage injections. The antisense Hex morpholino oligo (HexMO, 80 ng) has been described previously (Smithers and Jones, 2002). Synthetic RNA for microinjection was transcribed using the Message Machine Kit (Ambion) and purified on Microspin-6 columns (BioRad). The following plasmids were used for mRNA synthesis or directly for microinjection (total amounts of DNA or RNA injected, and enzymes used to linearize DNA templates and synthesize RNA are indicated): pCSKA-Xwnt8 (250 pg) (Christian and Moon, 1993);

pCS2+pt- $\beta$ -catenin (250 pg) (Yost et al., 1996); pCS2+MT Xgsk3 $\beta$  (500 pg) (Yost et al., 1996); pcDNA3- $\Delta$ NXTcf3 (800 pg) (Molenaar et al., 1996); pRN3 GFP (100 pg; *Sfi*I, T3); pCS2+ $\beta$ -gal (100 pg; *Not*I, Sp6); pCS107 tDkk1 (500 pg; *Asc*I, Sp6); pCS2+HA-GR- $\Delta$ NLEF- $\beta$ CTA (800 pg) (Domingos et al., 2001); pCS107-Xvent1 (500 pg; *Asc*I, Sp6); pCS107-Xvent2 (500 pg; *Asc*I, Sp6); pT7TS-HA-Xhex (500 pg; *Bam*HI, T7); pCS2+XfoxA2 (500 pg; *Not*I, Sp6); pT7TSHA-GR- $\Delta$ NXTcf3 (800 pg; *Xba*I, T7); pT7TSHA-GR-Vent2 (800 pg; *Xba*I, T7).

### *Xenopus* transgenics and transient luciferase assays

A *Xenopus laevis* genomic  $\lambda$ -library was screened using standard procedures to isolate *hhex* genomic clones and a ~6kb fragment upstream of the ATG start codon (Accession No. EF059707) was subcloned into the pGFP3 vector to create the phhex-6kb:GFP plasmid used in transgenesis. Transgenic *Xenopus* embryos were generated using sperm nuclear transplantation as previously described (Kroll and Amaya, 1996).

Three *hhex* promoter fragments –6.0, –1.56 and –0.44 kb were PCR amplified and cloned into the pGL2-Basic luciferase vector (Promega). The *hhex*:luciferase promoter constructs (300 pg) were microinjected along with a pRL-TK renilla (Promega) control vector (25 pg). At gastrula stage, five injected embryos were pooled together and homogenized in 100  $\mu$ l of 100 mM Tris (pH 7.5) on ice, assayed for luciferase activity and normalized to renilla activity using standard kits (Promega). Each construct was assayed in triplicate.

### In situ hybridization and RT-PCR analysis

In situ hybridizations were performed as previously described (Costa et al., 2003) using the following probes: *for1* (Seo et al., 2002), *pdx1/xlhbbox8* (Wright et al., 1989), *nkx2.1* (Small et al., 2000), *endocut/darmin* (Costa et al., 2003), *hhex* and *ampb* (Zorn and Mason, 2001), *vent2* (Onichtchouk et al., 1998), *pfal1* (Jarikji et al., 2007), *amylase* and *elastase* (Horb and Slack, 2002).

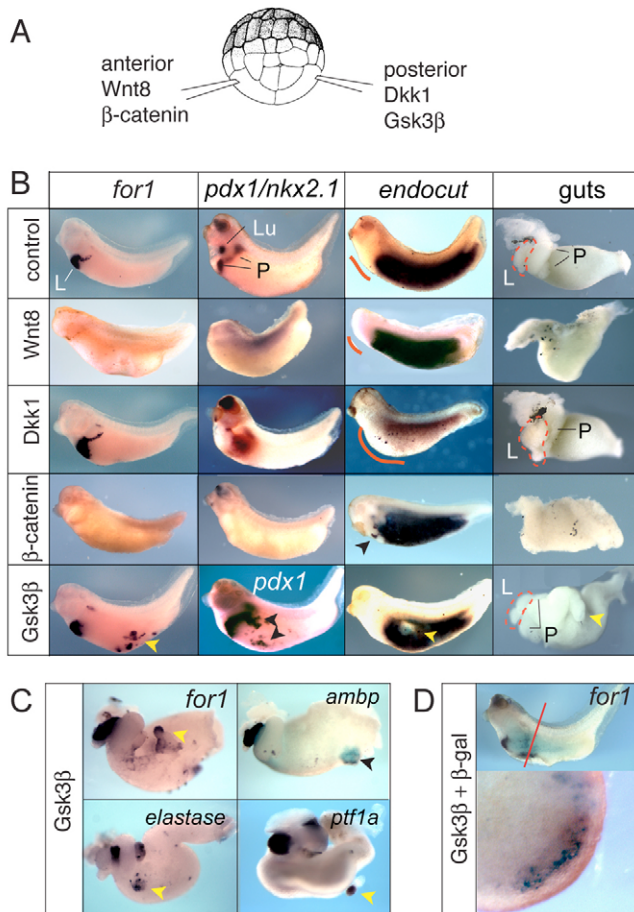
Real time RT-PCR analysis was performed on an Opticon PCR machine (MJ Research) as previously described (Sinner et al., 2004). The following primers were used (F, forward; R, reverse): *for1* (F, 5'-CTTA-ATGTGACTGAAGCAGAG-3' and R, 5'-TTTCCATCTGTAGAGC-CACAA-3'); *c-troponin* (F, 5'-AAGTCTCCATGGATCTAC-3' and R, 5'-CAGCTCTAACCATTTTCAG-3'); *endodermin* (F, 5'-AGCAGAA-AATGGCAAACACAC-3' and R, 5'-GGTCTTTTAATGGCAACAGGT-3'); *foxa2* (F, 5'-CCTATCATGAACCTCCTCATAG-3' and R, 5'-GGCC-AGAATACATACAGCAGTC-3'); *hhex* (Zorn et al., 1999); and *odc* (Sinner et al., 2004).

## RESULTS

### Repression of Wnt/ $\beta$ -catenin is necessary and sufficient to initiate liver and pancreas development

Maternal Wnt/ $\beta$ -catenin is active in the dorsal-anterior endoderm of the *Xenopus* blastula, but is dramatically downregulated at gastrulation (Schohl and Fagotto, 2002). It is this clearance of zygotic  $\beta$ -catenin in the anterior endoderm that we postulate is crucial to maintain foregut fate. To test this, we microinjected a Wnt8-plasmid into the D1 cells of 32-cell stage *Xenopus* embryos (Fig. 1A), which are fated to contribute to the foregut (Dale and Slack, 1987; Moody, 1987). The injected Wnt8 plasmid is not transcribed until the gastrula stage (Christian and Moon, 1993) and therefore only activates zygotic  $\beta$ -catenin signaling. At stages 35–37, when organ buds are first visible (Chalmers and Slack, 1998; Nieuwkoop and Faber, 1994), the embryos were assayed by in situ hybridization with the liver marker *for1* (Seo et al., 2002), as well as with markers for pancreas/duodenum (*pdx1/xlhbbox8*) (Wright et al., 1989), lung/thyroid (*nkx2.1*) (Small et al., 2000) and intestine (*endocut/darmin*) (Costa et al., 2003). In almost all of the treated embryos, foregut gene expression was inhibited and an examination of the gut tube at stage 42 revealed an absence of foregut organ buds in the Wnt8-injected embryos (Fig. 1B).

To determine if increased repression of Wnt signaling was sufficient to expand the foregut, we injected RNA encoding the canonical Wnt-antagonist Dkk1 (Glinka et al., 1998) into cells that contribute to the posterior-lateral endoderm (Dale and Slack, 1987; Moody, 1987). Dkk1 overexpression is known to inhibit zygotic but not maternal Wnt signaling (Glinka et al., 1998). The resulting embryos exhibited an expansion of *for1* and *pdx1* expression at the expense of the intestine marker, and at stage 42 the liver and pancreas buds were conspicuously enlarged (Fig. 1B).



**Fig. 1. Repression of  $\beta$ -catenin signaling in the endoderm is necessary and sufficient for liver and pancreas development.**

(A) 32-cell stage *Xenopus* embryos were injected with either a pCSKA-Wnt8 plasmid (250 pg) or stabilized pt- $\beta$ -catenin RNA (250 pg) in the D1 anterior endoderm cells. Other embryos were injected with RNA encoding Dkk1 (500 pg) or Gsk3 $\beta$  (500 pg) into D4 posterior endoderm cells to repress Wnt signaling. (B) In situ hybridization at stage 35 with the liver marker *for1*, or with a combination of pancreas/duodenum marker *pdx1/xlhbox8* and the lung marker *nkx2.1*, or with the intestinal marker *endocut*. Some embryos were hybridized with just *pdx1*. Arrowheads indicate ectopic or repressed gene expression. The solid red line indicates the relative size of the foregut domain. Gut tubes were isolated at stage 42 to visualize organ bud morphology. The dashed red line outlines the liver bud. L, liver; P, pancreas; Lu, lungs. (C) In situ hybridization to Gsk3 $\beta$ -injected guts with liver markers *for1*, *ambp*, the early pancreas marker *ptf1a* and the exocrine pancreas marker *elastase*. (D) A sectioned embryo co-injected with Gsk3 $\beta$  and  $\beta$ -gal RNA shows  $\beta$ -gal-staining nuclei (blue) and *for1* expression (brown) localized to the endoderm.

As Wnt8 and Dkk1 are secreted, we next addressed whether their effects on the endoderm were direct, or secondary to changes in mesoderm patterning. To cell-autonomously activate Wnt signaling in the anterior endoderm we microinjected RNA encoding a stabilized form of  $\beta$ -catenin (Yost et al., 1996) into the D1 blastomeres at the 32-cell stage. The stabilized  $\beta$ -catenin should persist in the gastrula anterior endoderm long after the time when endogenous  $\beta$ -catenin is downregulated, and this resulted in the repression of foregut markers as well as ectopic intestinal gene expression in the foregut domain (Fig. 1B and see Table S1 in the supplementary material). Unlike Wnt8 plasmid injections, there was little or no effect on head development in  $\beta$ -catenin-injected embryos, consistent with this acting in the endoderm and not affecting mesoderm or neural tissue. Co-injection of  $\beta$ -galactosidase ( $\beta$ -gal) RNA as a lineage-tracer confirmed that the injections targeted the anterior endoderm (data not shown).

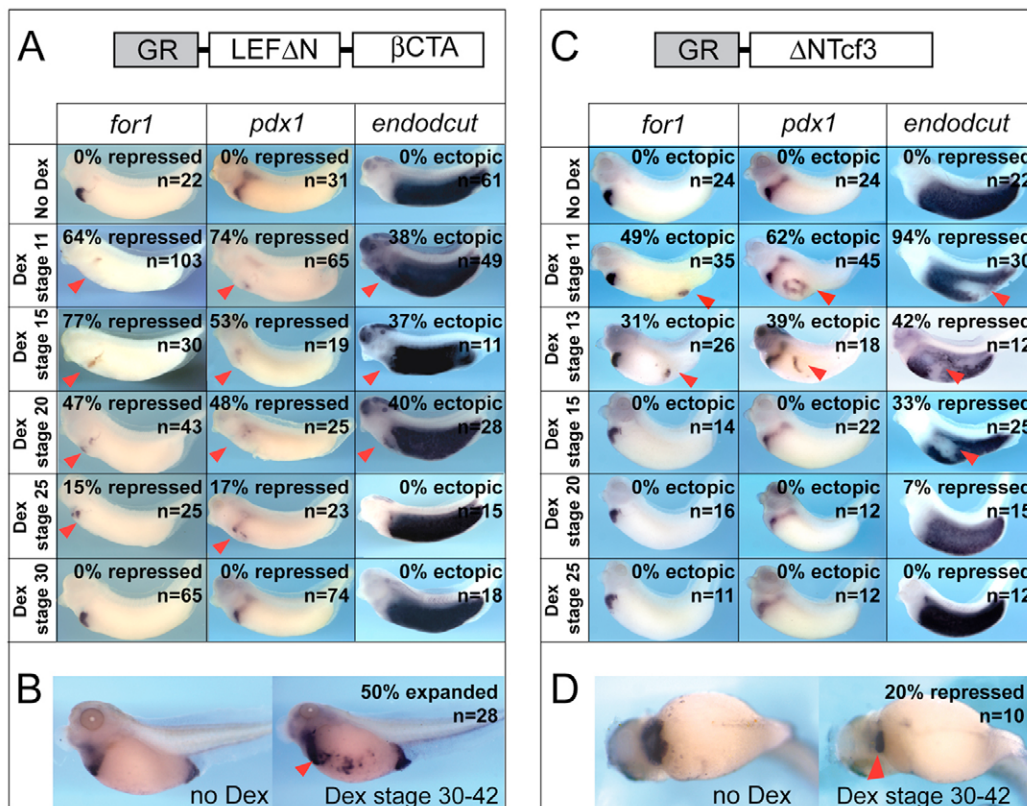
We next asked if reducing canonical Wnt signaling in the posterior endoderm was sufficient to induce foregut fate. To cell-autonomously repress  $\beta$ -catenin signaling we injected RNA encoding Gsk3 $\beta$  into the D4 posterior endoderm cells at the 32-cell stage. Gsk3 $\beta$  overexpression promotes  $\beta$ -catenin degradation (Yost et al., 1996) and as maternal  $\beta$ -catenin is not active in the ventral-posterior cells, Gsk3 $\beta$  is expected to repress zygotic  $\beta$ -catenin signaling. We observed ectopic *for1* and *pdx1* expression in the posterior endoderm of 50-60% of the Gsk3 $\beta$ -injected embryos, along with inhibition of the intestinal marker *endocut* (Fig. 1B and see Table S1 in the supplementary material). Similar results were obtained with a dominant-negative  $\Delta$ NTcf3 construct (Molenaar et al., 1996) that represses  $\beta$ -catenin/Tcf target genes (see Table S1 in the supplementary material). At stage 42-46, we observed ectopic organ buds in the intestines of Gsk3 $\beta$ - and  $\Delta$ NTcf3-injected embryos. Some of the ectopic buds expressed early liver and pancreas markers (*hhex*, *pdx1* and *ptf1a*) as well as hepatic (*for1*, *ambp* and *transferrin*) and exocrine pancreas (*elastase* and *amylase*) differentiation markers (Fig. 1C and see Table S1 in the supplementary material). However, we did not observe ectopic *insulin* expression suggesting that endocrine pancreas development requires additional signals.

To confirm that the injected Gsk3 $\beta$  acted autonomously in the endoderm, we co-injected Gsk3 $\beta$  with  $\beta$ -gal RNA as a lineage label. In none of the cases in which  $\beta$ -gal staining was restricted to the mesoderm ( $n=17$ ) was ectopic *for1* expression observed. By contrast, ectopic *for1* expression was observed in 19 cases in which the  $\beta$ -gal staining was in the endoderm and not the mesoderm (Fig. 1D), indicating that Gsk3 $\beta$  acts in the endoderm.

### **$\beta$ -catenin activity is repressed in the foregut during gastrula and early somite stages**

Since our injections did not disrupt head or axial mesoderm structures, this argues that we did not interfere with the known role of Wnt signaling in early axial patterning, which occurs during blastula and early gastrula stages. To rigorously test this, we repeated our experiments with hormone-inducible constructs that could be temporally controlled.

To confirm that  $\beta$ -catenin activity inhibits foregut fate after gastrulation, we used a GR-LEF $\Delta$ N- $\beta$ CTA construct, which has the hormone-binding domain of the glucocorticoid receptor fused to the Lef1 DNA-binding domain and the  $\beta$ -catenin transactivation domain. In the presence of dexamethasone (Dex), this fusion protein constitutively activates  $\beta$ -catenin target genes (Domingos et al., 2001). By treating GR-LEF $\Delta$ N- $\beta$ CTA-injected embryos with Dex at progressively later stages, we determined that  $\beta$ -catenin signaling



**Fig. 2. Temporal regulation of  $\beta$ -catenin/Tcf activity during endoderm patterning.** (A) At the 32-cell stage, *Xenopus* embryos were injected in the anterior D1 cells with RNA encoding the fusion protein GR-LEF $\Delta$ N- $\beta$ CTA (800 pg), which constitutively activates  $\beta$ -catenin target genes in the presence of dexamethasone (Dex). Dex (1  $\mu$ M) was added to the media of injected embryos at the indicated stages and embryos were assayed by *for1*, *pdx1* and *endodcut* in situ hybridization at stage 35. (B) Addition of Dex to GR-LEF $\Delta$ N- $\beta$ CTA-injected embryos from stage 30 to 42, followed by *hhex* in situ, revealed enlarged liver buds. (C) 32-cell stage embryos were injected in posterior D4 cells with RNA encoding GR- $\Delta$ NTcf3 (800 pg), which represses  $\beta$ -catenin/Tcf target genes when activated. Dex (1  $\mu$ M) was added to the media of injected embryos at the indicated stages and embryos were assayed by *for1*, *pdx1* and *endodcut* in situ hybridization at stage 35. (D) GR- $\Delta$ NTcf3 was injected into D1 cells at the 32-cell stage, and when Dex was added from stages 30 to 42 some embryos exhibited smaller liver buds based on *for1* in situ hybridization. No effect was observed in uninjected embryos treated with Dex.

must be excluded from the anterior endoderm between stage 11 (midgastrula) and stage 20 (6-7 somites). Dex treatment during this period inhibited *for1* and *pdx1* expression and expanded the intestinal marker into the foregut territory (Fig. 2A). Addition of Dex at stages 25 and 30 had no obvious effect on foregut development when assayed at stage 35. However, when Dex was added from stage 30 to 42, about half of the embryos exhibited enlarged liver buds (Fig. 2B). This is consistent with recent reports in zebrafish and mice suggesting that later in development,  $\beta$ -catenin promotes hepatoblast specification and/or proliferation (Ober et al., 2006; Tan et al., 2006).

To determine the window in time when ectopic foregut development could be induced in the posterior endoderm, we injected GR- $\Delta$ NTcf3 RNA into D4 blastomeres at the 32-cell stage. GR- $\Delta$ NTcf3 consists of the glucocorticoid receptor ligand-binding domain fused to a dominant-negative Tcf3, which represses  $\beta$ -catenin/Tcf target genes in the presence of Dex (Darken and Wilson, 2001). Activation of GR- $\Delta$ NTcf3 at midgastrula (stage 11) or early neurula (stage 13) efficiently induced ectopic *for1* and *pdx1* expression (Fig. 2C), whereas addition of Dex between stages 15 and 20 had no effect. When GR- $\Delta$ NTcf3 was injected into D1 anterior endoderm cells and Dex was added between stages 30 and 42, some embryos exhibited smaller liver buds (Fig. 2D) consistent

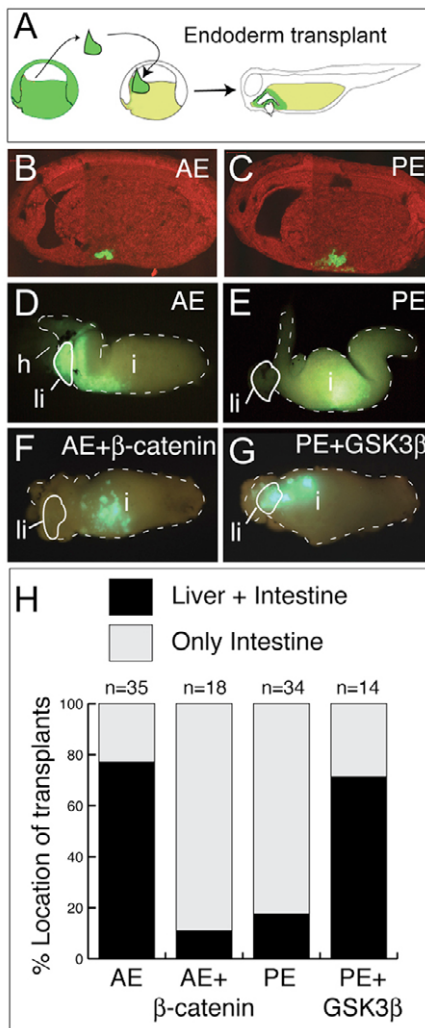
with the late induction of GR-LEF $\Delta$ N- $\beta$ CTA. These experiments reveal a  $\beta$ -catenin function that is distinct from its role in early axial patterning.

We conclude that between the gastrula and early somite stages, repression of  $\beta$ -catenin activity in the endoderm is necessary and sufficient to initiate liver and pancreas development, probably by regulating the formation of early foregut progenitors. However, during later embryogenesis,  $\beta$ -catenin has a second role in promoting hepatic development.

### The endoderm is a direct target of $\beta$ -catenin signaling

Although the lineage-tracing experiments suggested that  $\beta$ -catenin signaling acts directly on the endoderm to regulate lineage commitment, it was important to rigorously test this. We therefore isolated anterior endoderm from gastrula embryos injected with  $\beta$ -catenin and GFP RNA or posterior endoderm injected with Gsk3 $\beta$  and GFP RNA. This tissue was transplanted into the blastocoel of uninjected sibling host embryos and the fate of the cells was determined at stage 42 (Fig. 3A). By the neurula stage, the transplanted cells had incorporated into the host endoderm near the foregut/hindgut boundary (Fig. 3B,C). At stage 42, control transplants injected with GFP only respected their original anterior-

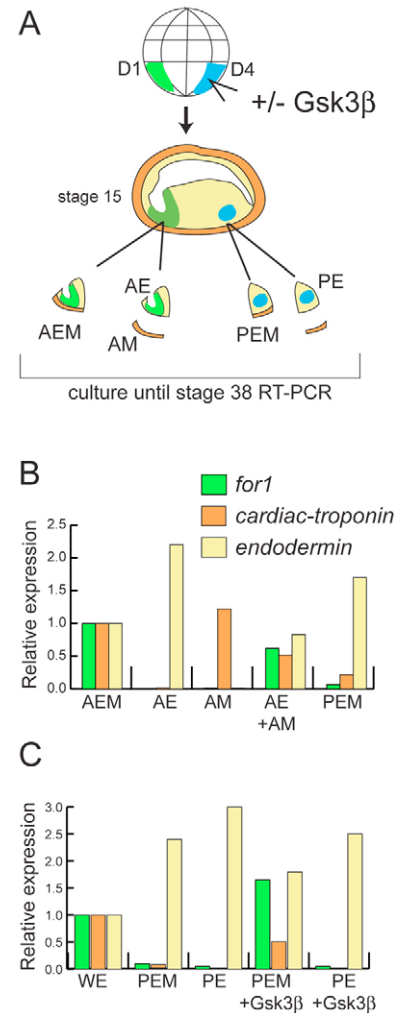
posterior identity. Anterior endoderm was incorporated in the liver and anterior intestine ~80% of the time, whereas control posterior endoderm transplants contributed to the intestine but rarely to the liver. By contrast, transplanted anterior endoderm overexpressing  $\beta$ -catenin almost never contributed to the liver, whereas posterior endoderm tissue injected with Gsk3 $\beta$  behaved like anterior endoderm and populated the liver bud ~80% of the time (Fig. 3D-H). Importantly, GFP-labeled cells were not observed in the heart or other mesoderm tissue demonstrating that differential  $\beta$ -catenin activity in the endoderm directly restricts regional identity.



**Fig. 3. The endoderm is a direct target of  $\beta$ -catenin signaling.** (A) Experimental design of the endoderm transplantations. Anterior endoderm (AE) or posterior endoderm (PE) was dissected from gastrula *Xenopus* embryos injected with RNA encoding GFP, pt- $\beta$ -catenin+GFP or Gsk3 $\beta$  and GFP and the tissue was transplanted into the blastocoel of uninjected sibling host embryos. (B,C) Confocal analysis at the neurula stage indicates that the GFP-labeled cells were incorporated into the host endoderm near the presumptive midgut. (D-G) At stage 42, control AE transplants (D) contributed primarily to liver (li) and foregut, whereas control PE transplants (E) mostly contributed to the intestine (i). By contrast, AE injected with pt- $\beta$ -catenin (F) rarely contributed to foregut, whereas PE injected with Gsk3 $\beta$  (G) frequently contributed to the liver. GFP-labeled cells were only observed in the endoderm and not the heart (h) or other mesoderm tissue. (H) Bar chart showing the location frequency of each type of transplant.

### An interaction with cardiogenic mesoderm is required for liver specification

We occasionally observed ectopic cardiac gene expression in the mesoderm adjacent to Gsk3 $\beta$ -injected posterior endoderm (see Table S1 in the supplementary material), consistent with a recent report that Wnt-antagonists promote cardiogenesis indirectly by acting on the endoderm (Foley and Mercola, 2005). Since cardiac mesoderm induces liver fate in mouse and chick, we asked whether the mesoderm was also required for normal and Gsk3 $\beta$ -induced liver specification in *Xenopus*. We isolated foregut explants at stage 18 (3-



**Fig. 4. The cardiogenic mesoderm is required for liver specification.** (A) At the 32-cell stage, *Xenopus* embryos were injected with Gsk3 $\beta$  RNA (500 pg) into the D4 posterior endoderm cell. At stage 18, endoderm explants were isolated from control and injected embryos, and cultured with or without their associated mesoderm (orange) until stage 35 when they were assayed by RT-PCR. Foregut explants from uninjected embryos were either left intact with the anterior endoderm and associated mesoderm (AEM), or the anterior endoderm (AE, green) was separated from the mesoderm (AM). Posterior endoderm and mesoderm (PEM), or posterior endoderm without its associated mesoderm (PE), was also isolated from control and Gsk3 $\beta$ -injected embryos (PE+Gsk3 $\beta$ ). (B,C) Bar charts showing the normalized relative mRNA expression levels from RT-PCR of the liver marker *for1*, heart marker *cardiac-troponin* and the pan-endodermal marker *endodermin*. WE, whole embryo; AE+AM, endoderm and mesoderm separated and immediately recombined.

4 somites) and cultured these with or without their associated cardiac mesoderm (Fig. 4A). RT-PCR analysis of the explants at stage 35 revealed that, as in mouse and chick, the mesoderm was required for liver gene expression (Fig. 4B). By removing the mesoderm at progressively later times, we found that liver specification requires an interaction with the mesoderm until stage 28 (20–22 somites) (data not shown). Similarly, an association with the adjacent mesoderm was also required for Gsk3 $\beta$ -injected posterior endoderm to express liver markers (Fig. 4C). We conclude that repression of  $\beta$ -catenin activity in the endoderm initiates a cascade of reciprocal signaling with the mesoderm leading to hepatic induction.

### The homeobox gene *hhex* mediates foregut development downstream of repression of Wnt

To determine whether low  $\beta$ -catenin activity establishes an early foregut domain, we examined the expression of the homeobox gene *hhex*, one of the earliest foregut markers (Newman et al., 1997;

Thomas et al., 1998) that is essential for early liver and ventral pancreas development in mice (Bort et al., 2004; Keng et al., 2000; Martinez Barbera et al., 2000).

Forcing  $\beta$ -catenin activity in the anterior endoderm by injection of either stabilized  $\beta$ -catenin or GR-LEF $\Delta$ N- $\beta$ CTA RNA resulted in a dramatic downregulation of *hhex* expression at stage 18 (Fig. 5A). Conversely, repressing  $\beta$ -catenin activity in the posterior endoderm by microinjecting Gsk3 $\beta$  plus  $\beta$ -gal RNA cell-autonomously induced ectopic *hhex* expression in the presumptive hindgut. A similar result was obtained by injecting either  $\Delta$ NTcf3 RNA or an antisense  $\beta$ -catenin morpholino oligo (Fig. 5A and see Table S1 in the supplementary material), indicating that low  $\beta$ -catenin activity is necessary and sufficient to induce early foregut progenitors.

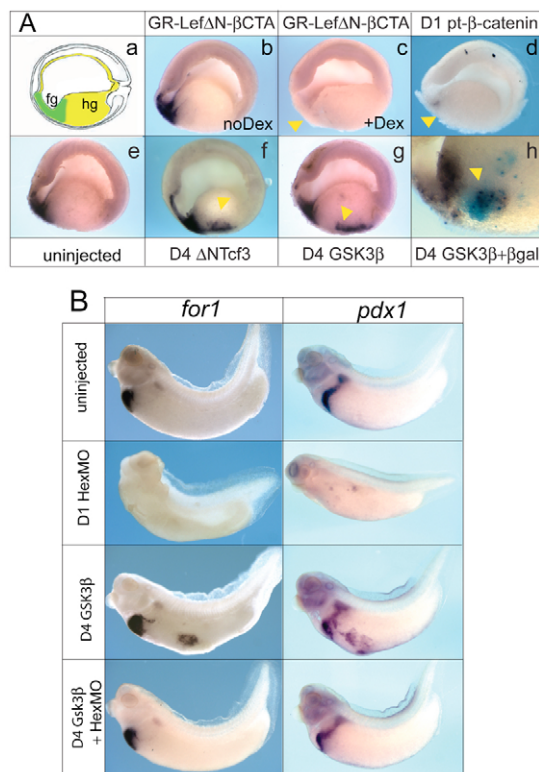
We also examined the expression of *foxa2* and *gata4*, *gata5* and *gata6* in  $\beta$ -catenin- and Gsk3 $\beta$ -injected embryos, as these transcription factors have been implicated in early mouse liver development (Lee et al., 2005; Zaret, 2002; Zhao et al., 2005). Although we did not observe obvious changes in *gata4-6* expression, *foxa2* foregut expression was downregulated by anterior  $\beta$ -catenin injection and modestly increased in the posterior endoderm by Gsk3 $\beta$  injection (see Fig. S1 in the supplementary material). Since the effects on *foxa2* expression were subtle, we focused our attention on *hhex* in our subsequent experiments.

We tested whether Hhex is required for liver and pancreas development in *Xenopus*. Embryos were injected with an antisense morpholino oligo complementary to the 5' untranslated region of the *hhex* mRNA (HexMO), which blocks translation and has been shown to specifically knockdown Hhex function (Smithers and Jones, 2002). HexMO-injected embryos exhibited very little if any *for1* or *pdx1* expression, demonstrating that Hhex is required for liver and pancreas development (Fig. 5B and see Table S1 in the supplementary material). Furthermore, injection of HexMO completely blocked the ectopic *for1* and *pdx1* expression induced by Gsk3 $\beta$  (Fig. 5B and see Table S1 in the supplementary material), indicating that Hhex function is required downstream of Gsk3 $\beta$ . Surprisingly, both the ventral and dorsal pancreas were absent from HexMO *Xenopus* embryos (as judged by *pdx1* expression), in contrast to the mouse *Hhex*<sup>-/-</sup> mutant in which only the ventral pancreas is compromised (Bort et al., 2004). The reason for this difference is not known, but we note that the anterior neural phenotype in HexMO *Xenopus* embryos is also more severe than in the mouse mutant (Smithers and Jones, 2002).

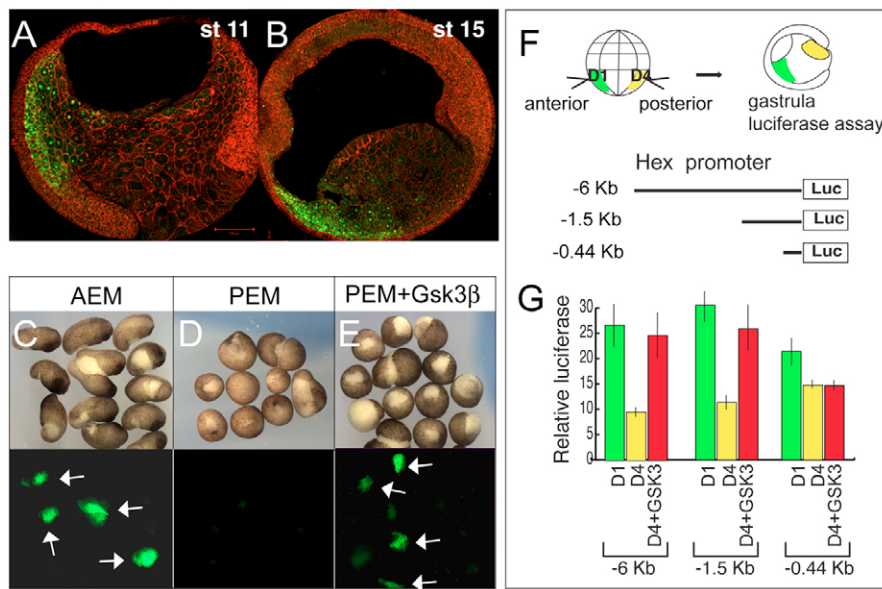
Finally, Hhex overexpressed in the posterior endoderm was not sufficient to induce ectopic *for1* or *pdx1* expression (data not shown), suggesting that additional factors are required to promote foregut development downstream of repression of Wnt.

### Regulation of *hhex* transcription

To examine how zygotic  $\beta$ -catenin represses *hhex* transcription, we isolated a -6 kb fragment of *Xenopus laevis* genomic DNA upstream of the *hhex* transcription start site and used this to generate germline *hhex:gfp* transgenic animals. Confocal analysis of gastrula- and presomitic-stage *hhex:gfp* transgenic embryos showed GFP fluorescence in the anterior endoderm and ventral foregut (Fig. 6A,B), recapitulating endogenous *hhex* expression. To determine if the -6 kb transgene was regulated by  $\beta$ -catenin, we injected Gsk3 $\beta$  into the posterior D4 cells of transgenic embryos, isolated hindgut explants at the gastrula stage and scored these for GFP at stage 18. As expected, GFP was observed in ~50% of control foregut explants (one parent was heterozygous for *hhex:gfp*) but was undetected in uninjected hindgut explants (Fig. 6C,D). By contrast, we observed



**Fig. 5. Regulation and function of *Xenopus hhex*.** (A) Analysis of *hhex* expression by in situ hybridization to bisected stage-18 embryos (anterior left). (a) Schematic of a stage-18 bisected embryo showing the presumptive foregut (fg, green) and hindgut domain (hg). (b) Injection of GR-LEF $\Delta$ N- $\beta$ CTA RNA (800 pg) into the D1 anterior endoderm cell has no effect without Dex. (c) Addition of Dex (1  $\mu$ M) at the midgastrula repressed *hhex* expression as does (d) D1 injection of stabilized pt- $\beta$ -catenin RNA (250 pg). (e) Uninjected control embryo. (f) Injection of  $\Delta$ NTcf3 RNA (800 pg) or (g) Gsk3 $\beta$  RNA (500 pg) in posterior D4 cells results in ectopic *hhex* expression (arrowhead). (h) Co-injection of Gsk3 $\beta$  and  $\beta$ -gal RNA reveals that the blue  $\beta$ -gal stain co-localizes with ectopic *hhex* in the endoderm. (B) Hhex is required for liver and pancreas development. 32-cell stage embryos were injected with either an antisense *hhex* morpholino oligo (HexMO, 80 ng) in the D1 cells or with Gsk3 $\beta$  or Gsk3 $\beta$  plus HexMO in D4 cells. At stage 35, embryos were assayed by in situ hybridization with liver (*for1*) or pancreas/duodenum (*pdx1*) probes.



**Fig. 6. Analysis of the *Xenopus hhx* promoter.** (A, B) Confocal analysis of bisected *hhx:gfp* transgenic embryos at (A) midgastrula and (B) early somite stages (anterior left), showing GFP (green) in the anterior endoderm. Embryos were counterstained with anti- $\beta$ -catenin antibodies (red). (C–E) ~50% of the foregut explants (AEM) isolated from embryos with a heterozygous *hhx:gfp* transgenic father were GFP-positive as expected (C), whereas posterior explants (PEM) did not express GFP (D). By contrast, 4 of 12 posterior explants isolated from sibling embryos injected with Gsk3 $\beta$  RNA (500 pg) were GFP-positive (E). Upper panels, bright field images; bottom panels, GFP. (F) The indicated *hhx:luciferase* constructs were injected into D1 or D4 cells at the 32-cell stage and luciferase activity was assayed at the gastrula stage. (G) Bar chart showing results of F as normalized relative luciferase activity.

ectopic GFP in 4 of 12 Gsk3 $\beta$ -injected hindgut explants (Fig. 6E), indicating that the  $-6$  kb promoter contains elements that confer correct A-P expression and Gsk3 $\beta$  responsiveness.

To better resolve the Gsk3 $\beta$ / $\beta$ -catenin-responsive elements, we microinjected plasmids containing deletion fragments of the *hhx* promoter driving luciferase expression (*hhx:luciferase*) into either D1 or D4 cells of 32-cell embryos (Fig. 6F) and the relative luciferase activity was determined at stage 11. The  $-6$  kb and  $-1.56$  kb reporters were highly expressed in the D1 anterior endoderm, but were much less active in posterior D4 cells. Gsk3 $\beta$  injection robustly stimulated the  $-6$  kb and  $-1.56$  kb constructs in the posterior endoderm. By contrast, the  $-0.44$  kb reporter was more active in the posterior endoderm and was not stimulated by Gsk3 $\beta$  (Fig. 6G). This indicates that promoter elements between  $-1.56$  and  $-0.44$  kb repress *hhx* transcription in the posterior endoderm and mediate Gsk3 $\beta$  responsiveness.

### Repression of *hhx* is mediated by Vent homeodomain factors

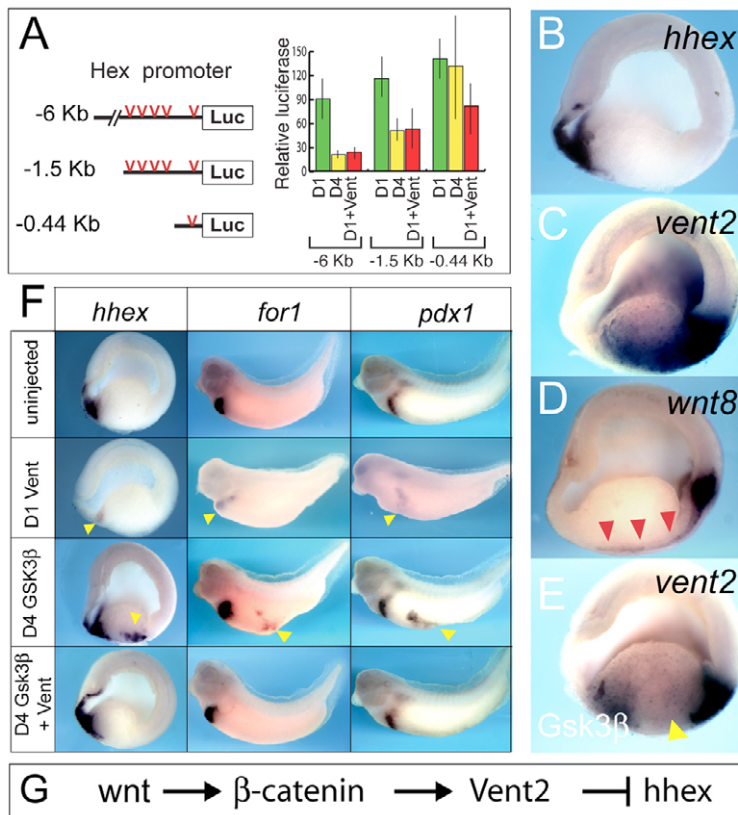
Because  $\beta$ -catenin-Tcf complexes normally stimulate transcription, we postulated that *hhx* expression must be repressed indirectly, by activating a transcriptional repressor. Sequence analysis of the *hhx* promoter revealed ten putative DNA-binding sites for the Vent class of homeodomain repressors (Friedle et al., 1998; Trindade et al., 1999), with eight of the Vent-sites located between  $-1.56$  and  $-0.44$  kb. Vent1 (PV.1) and Vent2 (Xbr-1, Vox and Xom) are closely related transcriptional repressors, best known as mediators of BMP signaling during dorsal-ventral mesoderm patterning (Ault et al., 1996; Onichtchouk et al., 1998; Papalopulu and Kintner, 1996; Schmidt et al., 1996). Although a function for Vents in the endoderm has not been described, recent evidence suggests that Wnt signaling promotes *vent* transcription in the mesoderm (Friedle and Knochel, 2002; Ramel and Lekven, 2004).

To test whether Vents could repress the *hhx* promoter, we injected the *hhx:luciferase* reporter constructs into D1 anterior cells, with or without RNA encoding Vent1 or Vent2, and assayed luciferase activity. Co-injection of either Vent repressed the activity of the  $-6.0$  kb and  $-1.56$  kb reporters to levels observed in the posterior endoderm (Fig. 7A; data not shown), whereas the  $-0.44$  kb promoter, lacking eight of the Vent-binding sites, was not efficiently

repressed. Thus, the  $-1.56$  to  $-0.44$  kb *hhx* promoter region exhibits all three activities: (1) it mediates *hhx* repression in the posterior endoderm; (2) it can be activated by Gsk3 $\beta$ ; and (3) it can be repressed by Vents.

Next, we re-examined the *vent1* and *vent2* expression patterns by in situ hybridization of bisected embryos, which exposes the deep endoderm. In addition to their previously documented mesodermal expression, we found that *vent2* (and to a lesser extent *vent1*) was highly expressed in the posterior endoderm during gastrula and early somite stages in a pattern reciprocal to *hhx*. Moreover, the *vent2*-expressing endoderm was adjacent to *wnt8*-expressing lateral plate mesoderm throughout this period of development (Fig. 7B–D and see Fig. S2 in the supplementary material). To test whether *vent2* expression in the posterior endoderm required Wnt/ $\beta$ -catenin activity, we injected either Gsk3 $\beta$ ,  $\Delta$ NTcf3 or antisense  $\beta$ -catenin morpholino oligos into the D4 posterior cells, all of which reduced *vent2* mRNA levels (Fig. 7E and see Table S1 in the supplementary material). Injection of Vent2 RNA into the D1 anterior endoderm at the 32-cell stage inhibited *hhx* and *foxa2* expression as well as subsequent liver and pancreas organogenesis (Fig. 7F; see Fig. S2 and Table S1 in the supplementary material). In addition, co-injection of Vent2 plus Gsk3 $\beta$  RNA in the D4 posterior endoderm blocked the ectopic *hhx*, *for1* and *pdx1* expression induced by Gsk3 $\beta$  alone (Fig. 7F and see Table S1 in the supplementary material).

Although we did not observe any changes in axial mesoderm development in these experiments, it was important to determine whether we were observing a Vent2 function distinct from its known role in gastrula-stage mesoderm patterning. We therefore repeated the experiments with a hormone-inducible GR-Vent2 construct that we could activate after early mesoderm patterning. We found that *for1* and *pdx1* expression was repressed even when the GR-Vent2 construct was activated by Dex as late as stage 20 (see Fig. S2 in the supplementary material). This indicates that Vent2 has a novel function in the endoderm well beyond the gastrula stage. Together, these data suggest a model in which Wnt/ $\beta$ -catenin activity promotes *vent2* transcription in the posterior endoderm during gastrula and early somite stages, and Vent2 protein then represses *hhx* transcription and foregut fate in the presumptive intestine (Fig. 7G).



**Fig. 7. Vent2 mediates  $\beta$ -catenin function.** (A) *Xenopus* embryos were injected with the indicated *hhex*:luciferase constructs with or without Vent2 RNA (500 pg) in D1 anterior or D4 posterior cells at the 32-cell stage. The bar chart shows the normalized relative luciferase activity at gastrula stage, indicating that Vent2 represses the *hhex* promoter. (B–D) In situ hybridization of bisected stage-18 embryos with the probes indicated. (E) Injection of Gsk3 $\beta$  RNA (500 pg) in the posterior endoderm repressed *vent2* expression. (F) Embryos were injected at the 32-cell stage with Vent2 RNA in anterior D1 cells or in posterior D4 cells with either Gsk3 $\beta$  or Gsk3 $\beta$  plus Vent2, followed by in situ hybridization at stage 18 with *hhex*, and stage 35 with *for1* or *pdx1* probes. (G) These data suggest a molecular pathway in which Wnt/ $\beta$ -catenin signaling promotes *vent2* expression and Vent2 represses *hhex* transcription.

## DISCUSSION

### A model of endoderm patterning and foregut development

We have identified a molecular pathway linking endoderm patterning to the initiation of liver and pancreas development in *Xenopus*. Our data supports a model in which the anterior-posterior axis of the endoderm is patterned by differential  $\beta$ -catenin activity during gastrula and early somite stages (Fig. 8). Low  $\beta$ -catenin in the anterior endoderm maintains foregut fates, whereas high  $\beta$ -catenin in the posterior promotes intestinal development. Immunostaining studies indicate that an anterior-to-posterior gradient of nuclear  $\beta$ -catenin is indeed present in *Xenopus* embryos (Schohl and Fagotto, 2002).

Our model predicts that Wnt ligands in the posterior mesoderm (probably Wnt8, Wnt8b and/or Wnt3a) signal to the adjacent endoderm to active  $\beta$ -catenin-Tcf complexes that promote expression of the homeobox gene *vent2*. The presence of Tcf-binding sites in the *vent2* promoter suggests that it might be a direct  $\beta$ -catenin/Tcf target (Friedle and Knochel, 2002; Rastegar et al., 1999). The transcriptional repressor Vent2 would then inhibit foregut fate by repressing *hhex* transcription, probably directly via Vent-binding sites in the *hhex* promoter. By contrast, the anterior endoderm secretes a number of Wnt-antagonists (Frzb1, Sfrp5, Crescent and Dkk1) that protect it from the Wnt ligands, resulting in low  $\beta$ -catenin levels and the expression of early foregut genes such as *hhex*. This appears to be essential for the development of early foregut progenitors (Deutsch et al., 2001), as both the liver and pancreas fates were affected in our experiments.

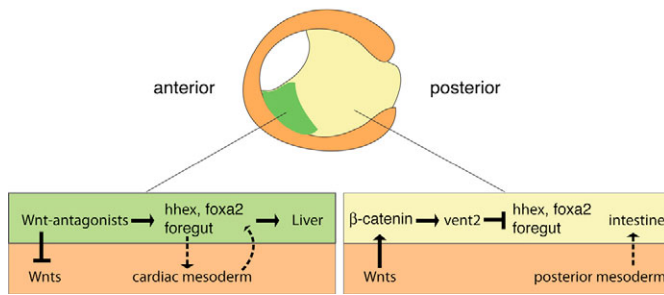
Our data also suggest that the repression of  $\beta$ -catenin activity is sufficient to initiate a cascade of reciprocal signaling that controls some, but not all, foregut organogenesis. For example, we observed

ectopic liver and exocrine pancreas development, but not endocrine pancreatic fates. In the case of the liver, it appears that low  $\beta$ -catenin activity imparts anterior character to the endoderm and this anteriorized endoderm then induces cardiac fate in the adjacent mesoderm (Foley and Mercola, 2005). The cardiogenic mesoderm then signals back to the foregut endoderm, instructing a subset of the cells to become liver (Zaret, 2002).

### Early targets of Wnt-mediated endoderm patterning

We found that Hhex was required, but not sufficient, for *Xenopus* foregut development. Although the direct targets of Hhex in this context are unknown, recent data in the *Xenopus* blastula suggest that Hhex acts in part by repressing the expression of the Groucho-family transcriptional co-repressor Tle4 (Zamparini et al., 2006). Repression of Tle4 by Hhex might also be important for maintaining the foregut precursors in a transcriptionally permissive state. However, as ectopic Hhex was not sufficient to initiate foregut development, other factors must also function downstream of the repression of Wnt. One candidate is the transcription factor Foxa2, which was also negatively regulated by  $\beta$ -catenin. Foxa2 is required for foregut development in mice (Dufort et al., 1998) and Zaret and colleagues have shown that Foxa binding to liver gene promoters correlates with hepatic competence (reviewed by Zaret, 2002). Consistent with this, liver development fails to initiate in mice in which *Foxa1* and *Foxa2* have been deleted from the endoderm (Lee et al., 2005). Another possibility is that even in the presence of Hhex or Foxa2, foregut development requires that intestine-promoting Wnt target genes such as Cdx genes must also be turned off. The Cdx homeobox genes are expressed in the posterior endoderm (and mesoderm), they are required for posterior development





**Fig. 8. A model of Wnt/ $\beta$ -catenin-mediated endoderm patterning in *Xenopus*.** Schematic showing an early-somite stage *Xenopus* embryo. Canonical Wnts secreted from the mesoderm (orange) signal to the adjacent posterior endoderm (yellow) to repress foregut development by activating the homeodomain repressor Vent2, which in turn inhibits the expression of key foregut genes such as *hhx* and *foxa2* in the posterior endoderm. The anterior endoderm (green) secretes a number of Wnt-antagonists that block the Wnt signals from the mesoderm, allowing *hhx* and *foxa2* expression to impart foregut identity. The *hhx*-expressing anterior endoderm then sends an unknown signal to the adjacent mesoderm inducing it to become cardiac. Later in development, the cardiogenic mesoderm signals back to the endoderm (curved dashed line), inducing a subset of the foregut endoderm to adopt a hepatic fate.

(Chawengsaksophak et al., 2004) and *Cdx1* has been shown to be a direct  $\beta$ -catenin/Tcf target in mouse and zebrafish (Lickert et al., 2000; Shimizu et al., 2005).

### Similarities between neural and endoderm patterning

The model of endoderm patterning that we present here has striking similarities to Nieuwkoop's classical 'activation/transformation' model of neural patterning (Nieuwkoop, 1999). In this model, an initial 'activation' signal induces neural tissue of anterior character, followed by a subsequent 'transformation' signal that imparts posterior character to the neural tube in a graded fashion, with the highest signal generating the most-posterior fates. There is now good evidence that the posteriorizing signals are a combination of Wnts, FGFs and retinoic acid (Domingos et al., 2001; Kudoh et al., 2002; Sasai and De Robertis, 1997), and recent evidence, including our study, suggests that these pathways also regulate endoderm patterning. Wells and colleagues found that high levels of Fgf4 promote intestinal fate and repress foregut development in mice and avian embryos similar to the Wnt activity we have described here (Dessimoz et al., 2006; Wells and Melton, 2000). In zebrafish, BMP signaling has been implicated in regulating anterior-posterior endoderm patterning (Tiso et al., 2002) and retinoic acid appears to specify the position of the pancreatic domain in *Xenopus*, zebrafish and mice (Chen et al., 2004; Martin et al., 2005; Stafford et al., 2004). In the future it will be interesting to determine how the Wnt, FGF, BMP and retinoic acid pathways functionally interact in the endoderm.

### Wnt-mediated endoderm patterning in other vertebrates

Our results are consistent with classical tissue recombination experiments in chick, which have shown that from the definitive-streak stage, the anterior endoderm but not the posterior endoderm is competent to become liver (Fukuda-Taira, 1981; Le Douarin, 1975). Our interpretation is that the posterior endoderm had already received a Wnt signal to repress foregut fate, which is also consistent

with explant studies showing that the posterior axial mesoderm emits signals that inhibit liver development (Gualdi et al., 1996; Le Douarin, 1975).

Although it remains to be determined whether  $\beta$ -catenin patterns the endoderm in other vertebrates, some genetic studies are consistent with this model. First, *TOPGal* transgenic mice, which contain a transcriptional reporter of  $\beta$ -catenin/Tcf activity, indicate that the anterior definitive endoderm experiences little if any Wnt signaling (Merrill et al., 2004). In addition, mice lacking both *Tcf1* and *Tcf4* have hindgut defects and an expansion of the anterior intestinal tract (Gregorieff et al., 2004), and ectopic activation of Wnt signaling in later-stage lung and pancreas development results in ectopic intestinal tissue (Heller et al., 2002; Okubo and Hogan, 2004). The role of individual Wnt ligands or Wnt-antagonists in the endoderm has been difficult to determine in mice because of genetic redundancy. Mutations in *Wnt3* or *Wnt3A* result in posterior truncations (Liu et al., 1999; Yamaguchi et al., 1999), whereas deletion of *Dkk1* results in anterior truncations (Mukhopadhyay et al., 2001); however, the endoderm in these mutant embryos has not been described in detail. *Sfrp5* mutant mice do not exhibit foregut defects, but the overlapping expression patterns of *Dkk1*, *Sfrp5*, *Sfrp1*, *Sfrp2* and *Frzb* (*Sfrp3*) suggest that they might act redundantly to repress  $\beta$ -catenin in the anterior endoderm (Finley et al., 2003; Heller et al., 2002; Kemp et al., 2005; Leaf et al., 2006).

### $\beta$ -catenin has multiple roles during hepatic development

The results we describe here, together with previously published reports, indicate that Wnts regulate multiple events in the hepatic lineage. Our previous studies have shown that the maternal Wnt pathway stimulates the initial expression of *hhx* and Wnt-antagonists in the anterior mesendoderm of the *Xenopus* blastula (Zorn et al., 1999). At this stage,  $\beta$ -catenin and *hhx* appear to cooperate to promote the formation of the anterior organizer (Zamparini et al., 2006). Now we show that just hours later, between the gastrula and early somite stages, zygotic  $\beta$ -catenin must be repressed to allow *hhx* expression and foregut development. Then, by stage 30, the function of  $\beta$ -catenin once again changes and it appears to enhance liver development. This later effect is consistent with recent reports that *Wnt2bb* is required for liver specification in zebrafish (Ober et al., 2006) and that  $\beta$ -catenin signaling promotes hepatoblast proliferation in the mouse and chick liver bud (Hussain et al., 2004; Tan et al., 2006). Finally, the inappropriate activation of  $\beta$ -catenin is linked to liver cancer (Park et al., 2001). Thus, one of the keys to understanding foregut organ development is to determine when a signaling pathway has to be turned on or off in a lineage and to characterize the resulting genetic program initiated in each case.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/12/2207/DC1>

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**Table S1. Summary of injection experiments**

Injection	Cells injected	Marker analysis	Number of embryos	
pt- $\beta$ -catenin (0.25 ng)	Anterior D1	51% repressed <i>for1</i>	n=70	Stage 35
		53% repressed <i>pdx1</i>	n=72	Stage 35
		32% repressed <i>nkx2.1</i>	n=65	Stage 35
		44% repressed <i>c-troponin</i>	n=9	Stage 35
		74% repressed <i>hhex1</i>	n=46	Stage 18
		41% repressed <i>foxa2</i>	n=22	Stage 18
		39% ectopic <i>vent2</i>	n=26	Stage 18
Gsk3 $\beta$ (0.5 ng)	Posterior D4	52% ectopic <i>for1</i>	n=90	Stage 35
		64% ectopic <i>pdx1</i>	n=58	Stage 35
		21% ectopic <i>nkx2.5</i>	n=19	Stage 35
		10% ectopic <i>nkx2.1</i>	n=20	Stage 35
		40% ectopic <i>ambp</i>	n=5	Stage 42
		44% ectopic <i>elastase</i>	n=9	Stage 42
		0% ectopic <i>insulin</i>	n=13	Stage 42
		60% ectopic <i>amylase</i>	n=5	Stage 42
		75% ectopic <i>ptf1a</i>	n=8	Stage 42
		49% ectopic <i>hhex</i>	n=220	Stage 18
		46% increased <i>foxa2</i>	n=59	Stage 18
		71% repressed <i>endocut</i>	n=17	Stage 35
		47% repressed <i>vent2</i>	n=47	Stage 18
		$\Delta$ NTcf3 (0.8 ng)	Posterior D4	50% ectopic <i>for1</i>
69% ectopic <i>pdx1</i>	n=13			Stage 35
52% ectopic <i>hhex</i>	n=21			Stage 18
87% repressed <i>vent2</i>	n=16			Stage 18
$\beta$ -catenin-MO (40 ng)	Posterior D4	49% ectopic <i>hhex</i>	n=33	Stage 18
		68% repressed <i>vent2</i>	n=37	Stage 18
Gsk3 $\beta$ (0.5 ng) +Hex-MO (80 ng)	Posterior D4	0% ectopic <i>for1</i>	n=45	Stage 35
		0% ectopic <i>pdx1</i>	n=34	Stage 35
Hex-MO (80 ng)	Anterior D1	100% repressed <i>for1</i>	n=28	Stage 35
		86% repressed <i>pdx1</i>	n=14	Stage 35
Gsk3 $\beta$ (0.5 ng) +Vent2 (0.5 ng)	Posterior D4	0% ectopic <i>for1</i>	n=39	Stage 35
		5% ectopic <i>pdx1</i>	n=22	Stage 35
		4% ectopic <i>hhex</i>	n=57	Stage 18
Vent2 (0.5 ng)	Anterior D1	47% repressed <i>for1</i>	n=27	Stage 35
		54% repressed <i>pdx1</i>	n=31	Stage 35
		63% repressed <i>hhex</i>	n=62	Stage 18
		47% repressed <i>foxa2</i>	n=15	Stage 18