Interplay between Wnt2 and Wnt2bb controls multiple steps of early foregut-derived organ development

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SUMMARY
The vertebrate liver, pancreas and lung arise in close proximity from the multipotent foregut endoderm. Tissue-explant experiments uncovered instructive signals emanating from the neighbouring lateral plate mesoderm, directing the endoderm towards specific organ fates. This suggested that an intricate network of signals is required to control the specification and differentiation of each organ. Here, we show that sequential functions of Wnt2bb and Wnt2 control liver specification and proliferation in zebrafish. Their combined specific activities are essential for liver specification, as their loss of function causes liver agenesis. Conversely, excess wnt2bb or wnt2 induces ectopic liver tissue at the expense of pancreatic and anterior intestinal tissues, revealing the competence of intestinal endoderm to respond to hepatogenic signals. Epistasis experiments revealed that the receptor frizzled homolog 5 (fzd5) mediates part of the broader hepatic competence of the alimentary canal. fzd5 is required for early liver formation and interacts genetically with wnt2 as well as wnt2bb. In addition, lack of both ligands causes agenesis of the swim bladder, the structural homolog of the mammalian lung. Thus, tightly regulated spatiotemporal expression of wnt2bb, wnt2 and fzd5 is central to coordinating early liver, pancreas and swim bladder development from a multipotent foregut endoderm.

KEY WORDS: Wnt signalling, Liver, Swim bladder, Endoderm patterning, Organogenesis, Zebrafish

INTRODUCTION
A central focus in developmental biology is to understand the specific cellular and molecular programmes coordinating the intricate steps of organ formation, such as progenitor specification and their functional differentiation into specific cell types, as well as organ morphogenesis and growth. The liver forms in the context of the developing digestive system from the multipotent foregut endoderm, representing an excellent model to elucidate these issues. The nascent foregut gives rise not only to the liver, but also the lung, pancreas and alimentary canal tissue. The development of these organs requires signals from neighbouring tissues such as the lateral plate mesoderm (LPM) for the establishment of regional identity and specific organ fates along the anteroposterior body axis (Grapin-Botton, 2005). Although the molecular framework of digestive system organogenesis is beginning to emerge, many questions remain regarding the specific factors directing multipotent progenitors to a particular organ fate, their interactions and the evolution of their specific functions.

In zebrafish, the anlage of the digestive tract starts to form after gastrulation, when two endodermal sheets converge towards the midline into a single rod-like structure (Ober et al., 2003). The dorsal endocrine pancreas is the first organ to form at mid-somitogenesis stages (Biemar et al., 2001). Subsequently, hepatoblasts are specified anteriorly to the endocrine pancreas within the ventral foregut domain around 23 hours post fertilisation (hpf) (Field et al., 2003b). These hepatoblasts aggregate into a liver bud, which grows out to the left, and the subsequent formation of the extrahepatic duct connects the liver to the digestive tract at 48 hpf. The ventral pancreatic bud, which gives rise to exocrine and endocrine cells, appears around 34 hpf from a region posterior and close to the liver, and later merges with the dorsal pancreatic bud (Field et al., 2003a). At the same time the swim bladder, the structural homolog of the mammalian lung, arises from a domain anterior to the liver (Winata et al., 2009).

Subsequent to gross endoderm regionalisation, members of the Bone morphogenetic protein (Bmp), Fibroblast growth factor (Fgf) and Wnt families promote liver formation in the multipotent, ventral foregut endoderm (Zaret et al., 2008; Zorn, 2008). Wnt/β-catenin signalling in particular has been implicated in various steps of the formation of the alimentary canal and its accessory organs (Cardoso and Lu, 2006; Nejak-Bowen and Monga, 2008; Verzi and Shivdasani, 2008). Wnt ligands are secreted glycoproteins that regulate gene transcription by controlling the stability of the intracellular effector β-catenin (Clevers, 2006). Previous studies have shown that Wnt pathway components – including ligands, receptors and modulators – are expressed in restricted patterns along the anteroposterior axis of the developing digestive system of vertebrates and invertebrates, implying ‘code-like’ functions of these factors in patterning and differentiation of this system (Heller et al., 2002; Janson et al., 2001; Lickert et al., 2001; McBride et al., 2003; Theodosiou and Tabin, 2003). However, little is known about their specific roles within these processes. Embryological and genetic studies manipulating predominantly intracellular pathway components have revealed that Wnt/β-catenin signalling is required repeatedly for hepatic development in vertebrates (Nejak-Bowen and Monga, 2008), including initial formation of hepatoblasts, the liver progenitor cells that differentiate into hepatocytes and biliary epithelial cells (Goessling et al., 2008; McLin et al., 2007; Ober et al., 2006), and their subsequent survival and proliferation (Goessling et al., 2008; Tan et al., 2008), as well as hepatocyte differentiation (Decaens et al., 2008). Work in zebrafish and Xenopus laevis revealed a biphasic requirement for Wnt signalling in early hepatic development, showing that it needs to be repressed at the end of gastrulation to establish foregut identity, crucial for subsequent liver and pancreas organogenesis, and shortly after it

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stimulates liver formation (Goessling et al., 2008; McLin et al., 2007). In zebrafish prometheus (prt)/wnt2bb mutants, hepatoblast specification is impaired, as a reduced number of hepatoblasts form with a timely delay (Ober et al., 2006). Hepatoblast specification was assessed by expression of the transcription factors proxl and hhex, which represent two of the earliest genes expressed in the forming liver in zebrafish, mouse and chick (Burke and Oliver, 2002; Ober et al., 2003; Thomas et al., 1998; Zhang et al., 2004). In the majority of prt/wnt2bb mutant embryos these hepatoblasts give rise to a smaller liver bud and some mutants will develop into viable adults, suggesting that the liver can recover. wnt2bb is expressed in bilateral domains in the LPM adjacent to the presumptive hepatoblasts. Together, these data indicate that while wnt2bb is required for liver specification, other factors contribute to the formation of this vital organ.

Here, we show that the specific and overlapping activities of wnt2 and wnt2bb control hepatic specification and subsequent proliferation of newly specified liver progenitors. wnt2 and wnt2bb signalling is in addition pivotal for swim bladder formation, enabling us to identify two factors essential for this molecularly poorly understood developmental process. Gain-of-function analyses revealed that both ligands are sufficient to induce ectopic intestinal tissue, demonstrating that the ventral digestive tract endoderm posterior to the foregut is competent to respond to hepatic signals. This wider competence is mediated by the receptor frizzled homolog 5 (fd5). In summary, our study reveals that precisely regulated spatiotemporal expression of two specific Wnt ligands is essential for establishing foregut-derived organ fates of a more widely competent digestive tract, providing a robust mechanism that ensures the development of these vital organs.

MATERIALS AND METHODS

Fish stocks

Adult zebrafish and embryos were raised according to standard laboratory conditions (Westerfield, 2000). The following strains were used: wnt2/mouse:prt (Ober et al., 2006), Tg(XIeef1a:GFP)y834 (Field et al., 2003b), Tg(ptf1a:eGFP)y63 (Dong et al., 2007; Godinho et al., 2005), Tg(fabp10:dsRed6)y45, Tg(eala3l:EGFP)y92 (Dong et al., 2007) and wild type.

Following in situ hybridization or immunohistochemistry, wnt2/wnt2bb embryos were genotyped by PCR amplification (F:5'-GTTGTTGTGGGTGGATTCTTCTCCACCACTTCTTTGAAGGATGGAAGGAACTTTGAAGGAACGACATG-3'; R:5'-CCACCGATAGAAAAACCTGTCCA-3'), generating a PCR product of 596 bp from genomic DNA of whole embryos or tail tissues followed by digestion with FokI for wnt2/wnt2bb or NcoI for prt/wnt2bb. Each experiment was carried out with three alleles.

Immunohistochemistry

Immunostainings were carried out as previously described (Ober et al., 2006), using monoclonal antibodies against 2F11 (Abcam, 1:1000), Islet1/2 (Developmental Studies Hybridoma Bank, 1:200), anti-BrDU (G34, Developmental Studies Hybridoma Bank, 1:25), and polyclonal antibodies against Insulin (guinea pig, Biomena, 1:1000), Proxl (rabbit, Chemicon, 1:1000), GDF (rabbit, Torrey Pines Biolabs, 1:500), cleaved-Caspase3 (rabbit, ASPI75, Cell Signalling, 1:75). Fluorophore-conjugated secondary antibodies were obtained from Jackson Laboratories (USA). Embryos were mounted in 4% low-melting-point agarose/1% gelatin, sectioned at 130 μm using a Leica Vibratome and visualised using a Zeiss LSM5 Pascal Excit confocal microscope. Images were processed and numbers of cells were determined with Velocity image analysis software (Improvision).

In situ hybridisation

Whole-mount mRNA in situ hybridisation was performed as described (Ober et al., 2006). Embryos older than 24 hpf were treated with 0.2 mM 1-phenyl-2-thiourea in egg water to inhibit melanin production. Fluorescent mRNA in situ hybridisation was carried out using the TSA Plus Fluorescence palette system (Perkin-Elmer). Anti-digoxigenin-HRP (Roche) was used at 1:1000 and Tyramide-labelled substrate at 1:50. The following probes were used: ceruloplasmin (Korzh et al., 2001), foxa1 (Odenthal and Nüsslein-Volhard, 1998), hhex (Ho et al., 1999), wnt2 (Blader et al., 1996), wnt2bb (Ober et al., 2006), agr2 (Shih et al., 2007), pdx1 (Lin et al., 2004) and ptf1a (Lin et al., 2004).

BrDU treatments

BrDU (Sigma) was resuspended in DMSO at 100 mM and diluted in egg water at 10 mM in a final concentration of 15% DMSO. Embryos were incubated 30 minutes at 28°C in a shaking incubator. Fixed embryos were washed in PBST and incubated in 1 N HCl for 1 hour. Three washes in borate buffer (0.1 M, pH 8.5) allow pH recovery before proceeding to immunohistochemistry as described above.

Morpholino knockdown

Three different antisense morpholino oligonucleotides (MOs) against wnt2 (Gene Tools) were injected into one-cell stage embryos (see Fig. S1 in the supplementary material). All experiments were repeated at least three times. 3 ng MO acc-wnt2, 14 ng MO don-wnt2 and 3 ng MO ATG-wnt2 were injected per embryo. All experiments were carried out at least with MO acc-wnt2. The efficiency of the knockdown was tested by RT-PCR (see Fig. S1 in the supplementary material) after RNA extraction with TRIzol reagent (Invitrogen) from five embryos per condition. Per embryo, 7 ng MO-fds5 (Cavodeassi et al., 2005) were co-injected with 7 ng MO-p53 (standard MO, Gene Tools, LLC).

Generation and injection of wnt2 and wnt2bb overexpression constructs

An hsp70:mCherry-2A-wnt2bb construct was generated using a PCR-based strategy and the sequence verified. The 19 amino acid PTV1-2A motif was inserted between the coding sequences of mCherry and wnt2bb or wnt2 with added glycine-serine-glycine residues (Holst et al., 2006): GSGATNFSLLKQADVEENPGP. This cassette was placed under the control of the zebrafish hsp70-4 promoter and subsequently between minito12 sequences. hsp70-mCherry-2A was used as control construct. All constructs were co-injected with transposase mRNA (30 pg each/embryo); 1 hour heat shock at 39°C was carried out at indicated time points. In a subset of experiments, stable transgenic lines for both transgenes have been used. The generation of these lines will be described elsewhere (M.P. and E.A.O., unpublished results).

qPCR

Quantitative PCR (qPCR) was performed using primer sets for cycinDl, fabp10a and myca (cycDl 5'-GGCTAAAAATGAGAAAATCTG-3', cycDl 5'-CCACGATAGAAAAACCTGTCCA-3'; fabp10a 5'-CTC-CTGGAACCCGTACCAACA-3', fabp10aR 5'-TCCGAACCAGGCCTTCTCCA-3'; mycaF 5'-CACCTCACTAAGCATCCCTCA-3', mycaR 5'-CAATTCTCTCCCTCTCTCTCC-3'; wnt2F 5'-TGTGACGACCCAGGTTCACCAGC-3', wnt2R 5'-ACAAAGTGCTGCCAGCAGCT-3'; rpl13F 5'-TCTGGAGAGCTTAAGGATGC-3', rpl13R 5'-AGACGCGCAATCTTGAGGAGC-3'). Embryos were treated as described and mRNA was extracted either from whole embryos or foregut-containing trunk tissue. Tissue from 5-10 embryos was pooled and processed. qPCR (60°C annealing temperature) were carried out using Absolute SYBR Green Mix (Thermo) on the ABI Prism 7000 RTPCR Detection system. Expression values were normalized using rpl13 and compared by Student's t-test. Unless indicated differently, three independent experiments were assessed with three samples each.

RESULTS

Dynamic wnt2 expression in the LPM

To test a code-like activity of different Wnt ligands in digestive system development, we focused on the formation of the liver and its adjacent organs. Among the possible Wnt ligands that could control liver development and interact with the previously identified wnt2bb (Ober et al., 2006), wnt2 (Blader et al., 1996)
represented a good candidate. We uncovered its bilateral expression within the LPM adjacent to the liver forming endoderm (Fig. 1A), resembling \textit{wnt2bb} expression at this stage (see Fig. S1A in the supplementary material). Whereas \textit{wnt2bb} expression precedes the onset of \textit{hhex} and \textit{prox1} expression in newly specified hepatoblasts at 23 hpf (Ober et al., 2006), \textit{wnt2} expression was initiated shortly after 24 hpf. The bilateral \textit{wnt2} domains were found progressively closer to each other until they overlapped on the right side of the embryo, adjacent to the midline, at 30 hpf (Fig. 1B). Fluorescent detection of \textit{wnt2} in \textit{Tg}(XlEef1a1:GFP\textsuperscript{s854}) embryos, in which Green fluorescent protein (GFP) highlights the endoderm of the developing digestive system, revealed that at 30 hpf \textit{wnt2}-expressing LPM was located dorsolaterally to the endoderm, at the base of the outgrowing liver and the future swim bladder extending towards the dorsal pancreas (Fig. 1C). At 48 hpf, \textit{wnt2} was expressed in the mesoderm between the swim bladder and the forming pancreas (Fig. 1D,E). Thus, \textit{wnt2} is dynamically expressed in tissues adjacent to the prospective liver and swim bladder, suggesting possible roles for \textit{wnt2} in endodermal organogenesis. In particular, this expression makes it a candidate to cooperate with \textit{wnt2bb} in early liver development.

\textbf{Wnt2 is required for proliferation of the hepatic primordium}

The spatiotemporal expression of \textit{wnt2} led us to examine its function in hepatic development using a loss-of-function approach. Embryos were depleted of functional \textit{Wnt2} by injecting antisense MOs into one-cell-stage embryos. To ensure the specificity of the knockdown, we used three different MOs producing indistinguishable phenotypes (see Fig. S1B in the supplementary material). Injecting \textit{Tg}(XlEef1a1:GFP\textsuperscript{s854}) (\textit{G}), \textit{Tg}(ptf1a:eGFP\textsuperscript{l1}) (\textit{I}, \textit{K}, \textit{M}) embryos, stained for Prox1 and Islet1/2 or Insulin, respectively. The ventral, exocrine and endocrine pancreas serve as developmental landmarks; a subset of \textit{MO-wnt2} embryos show ectopic endocrine cells (L). (N-O) Ventral views; all anterior towards the top. *, endocrine pancreas; ex p, exocrine pancreas; L, liver; p, pancreas; sb, swim bladder. (P) Proliferation rates in controls, \textit{MO-wnt2} and \textit{prt/wnt2bb} embryos determined by Prox1 and BrdU labelling. Standard errors are shown. Asterisks indicate the $P$ value for each condition (*$P<0.05$; **$P<0.001$); \textit{n}, number of embryos. Data are mean±s.e.m.
Table 1. Quantification of hepatic phenotypes following wnt2 and wnt2bb deletion

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>Number of control embryos</th>
<th>Number of wnt2bb embryos with reduced liver</th>
<th>Percentage of wnt2bb embryos with reduced liver</th>
<th>Fold decrease of liver size</th>
<th>P-value for each experiment</th>
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</thead>
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<tr>
<td>Wild type</td>
<td>30 hpf</td>
<td>4</td>
<td>16</td>
<td>33.33</td>
<td>0.92*</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>34 hpf</td>
<td>25</td>
<td>38</td>
<td>30</td>
<td>0.93*</td>
<td>&gt;0.06</td>
</tr>
<tr>
<td></td>
<td>48-53 hpf</td>
<td>75</td>
<td>128</td>
<td>62.62</td>
<td>0.46*</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td></td>
<td>74 hpf</td>
<td>35</td>
<td>53</td>
<td>61.18</td>
<td>0.58*</td>
<td>1×10⁻⁶</td>
</tr>
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Average number of hepatic Prox1 cells was determined for each sample using Volocity software. Difference of liver size is shown as fold-decrease between control (wild type or prl/wnt2bb) and entire populations of experimental embryos (MO-wnt2 or MO-wnt2;prl/wnt2bb). *Determined by Prox1 positive hepatoblast number. **Determined by fabp10:dsRed volume.

apparent defects in exocrine pancreas development were observed in MO-wnt2 embryos. Quantifying hepatic Prox1 expression confirmed a mild liver reduction in MO-wnt2 embryos at 34 hpf, and a more severe reduction at 53 hpf (Table 1). Although the liver was reduced in size, expression of hepatic differentiation genes such as the plasma protein ceruloplasmin (cp) or dsRed expressed under the control of the fatty acid binding protein 10 (fabp10) promoter was not impaired (Fig. 2E,G,O,Q).

The reduction in liver size could be the result of a defect in the specification of liver progenitors, increased cell death or decreased cell proliferation rates. Analysis of hepatic hhex expression in MO-wnt2 embryos at 26 hpf revealed no obvious changes (data not shown), suggesting that wnt2 is not required for liver specification. Although a few dying cells could be detected in a small subset of embryos in the neighbouring LPM, no apparent cell death was observed in the hepatic foregut endoderm of MO-wnt2 embryos at 25 hpf (see Fig. S1C in the supplementary material). Likewise, coinjection of MO-wnt2 with MO-p53 uncovered no obvious change in the observed liver hypoplasia, indicating that p53-mediated cell death is unlikely to contribute to this phenotype (data not shown).

Conversely, 5-bromo-2-deoxyuridine (BrdU) incorporation assays revealed that cell proliferation rates in the developing liver of MO-wnt2 embryos at 26 hpf were similar to controls, but at 30 hpf were reduced by 18% compared with controls (Fig. 1N-P). These data indicate that wnt2 is required for promoting hepatoblast proliferation, consistent with an increasing reduction of liver size from 30 hpf onwards. Consistent with the observation that wnt2bb is expressed in a domain similar to wnt2, BrdU assays carried out at 30 hpf revealed a 41% reduction in proliferation in prl/wnt2bb mutants compared with controls (Fig. 1P). Moreover, proliferation of the LPM adjacent to the liver was reduced by 32% in prl/wnt2bb embryos (n=18; see Fig. S3I in the supplementary material) and not consistently affected in MO-wnt2 (n=17) at this stage, suggesting that Wnt2bb might have additional functions in the LPM. Altogether, these findings indicate sequential functions for wnt2 and wnt2bb in early liver formation. First, wnt2bb promotes hepatoblast specification (Ober et al., 2006) and subsequently, wnt2 and wnt2bb are required for hepatoblast proliferation.

**Combined activity of Wnt2 and Wnt2bb is essential for liver formation**

To determine whether Wnt2 may interact with Wnt2bb, MO-wnt2 was injected into prl/wnt2bb mutants. Although loss of Wnt2bb or Wnt2 alone resulted in reduced hepatic hhex expression at 28 hpf (100% of prl/wnt2bb, n=26; 37% of MO-wnt2, n=40), this expression domain was absent in 91% and reduced to few cells in 7% of MO-wnt2;prl/wnt2bb embryos (n=57; Fig. 2A-D). Consistently, no Prox1-positive cells were observed in MO-wnt2;prl/wnt2bb embryos at 30 hpf (n=6; data not shown). At 52 hpf, the majority of MO-wnt2;prl/wnt2bb embryos still exhibited a loss of Prox1 expression in the presumptive hepatic domain, whereas Prox1 expression in the exocrine pancreas was maintained (Fig. 2L,‘L’,‘L’; see Fig. S2E in the supplementary material; Table 1). Absence of cp expression at 48 hpf, confirmed the loss of hepatic tissue in MO-wnt2;prl/wnt2bb embryos (100%, n=8), compared with reduced domains in prl/wnt2bb (100%, n=6) and MO-wnt2 (64%, n=25; Fig. 2E-H). Consistently, hepatic Tg(fabp10:dsRed) expression at 74 hpf was absent in 80% or severely reduced in 20% in MO-wnt2;prl/wnt2bb embryos (n=25; Fig. 2O-R). This phenotype persists, as few to no cells expressing hepatic Tg(fabp10:dsRed) were detected in 69% of MO-wnt2;prl/wnt2bb embryos at 6 dpf (n=13; see Fig. S2A-D in the supplementary material). This indicates that Wnt2 partially compensates for the loss of Wnt2bb in liver formation. To determine whether this includes an increase of wnt2 expression in prl/wnt2bb embryos, whole-mount mRNA in situ hybridisation and qPCR analysis were carried out at 26 hpf, shortly after its onset of expression and before hepatoblasts are detected in prl/wnt2bb mutants. These experiments showed no significant increase in the domain and levels of wnt2 expression in prl/wnt2bb embryos (data not shown), excluding the possibility that increased wnt2 expression compensates for loss of Wnt2bb and further suggesting that Wnt2bb does not regulate wnt2 in this context. These data show that Wnt2 and Wnt2bb are key secreted signals regulating liver specification.

Strikingly, in 58% of MO-wnt2;prl/wnt2bb embryos (n=65), absence of the liver was accompanied by a reduction of the adjacent extrahepato-pancreatic ductal system (EHPD), which connects the liver and pancreas to the alimentary canal and is visualised by strong 2F11 expression (Fig. 2L,‘L’-N). The extent of the forming EHPD varies between MO-wnt2;prl/wnt2bb embryos, from a mild reduction (Fig. 2L) to an almost complete loss of EHPD, including the prospective gall bladder (Fig. 2L,N,O,R). The progressive increase in phenotypic severity of MO-wnt2;prl/wnt2bb could reflect a dosage-dependent requirement for Wnt2 and Wnt2bb signalling in liver and EHPD precursor formation. In addition, in a subset of these embryos we observed Prox1-positive organ bud(s) in ectopic positions (e.g. presumptive
ventral pancreas on the left side, separated from the dorsal pancreatic bud), possibly indicating morphogenesis defects (Fig. 2I,N). Pancreatic Tg(XlEef1a1:GFP)sz2 expression at 74 hpf verified the identity of these Prox1-positive buds in MO-wnt2;prt/wnt2bb (Fig. 2O,R). To quantify these phenotypes, the number of Prox1-positive cells in the hepatic and pancreatic domains was determined in MO-wnt2, prt/wnt2bb and MO-wnt2;prt/wnt2bb embryos at 48 hpf (see Fig. S2E in the supplementary material), respectively showing a continuous decrease of Prox1 cells in the hepatic domain. By contrast, the number of pancreatic Prox1 cells was increased in prt/wnt2bb and MO-wnt2;prt/wnt2bb embryos and unchanged in MO-wnt2. The increase of pancreatic cells in prt/wnt2bb embryos was statistically significant, whereas the increase in MO-wnt2;prt/wnt2bb was slightly smaller and not significant. Moreover, qPCR showed reduced levels of fabp10a in prt/wnt2bb, MO-wnt2 and MO-wnt2;prt/wnt2bb embryos (see Fig. S2F in the supplementary material), confirming our previous results.

Our findings indicate that wnt2 compensates partially for loss of wnt2bb function in prt/wnt2bb mutants. (A-H) Expression of hhex at 28 hpf (A-D) and ceruloplasmin at 48 hpf (E-H) shows a reduction of liver progenitors (bracket) in embryos lacking either wnt2 (C,G) or wnt2bb (B,F) and an absence in MO-wnt2;prt/wnt2bb embryos (D,H), whereas the dorsal pancreas is unaffected (asterisk). (I-N) Prox1 expression in Tg(XlEef1a1:GFP)sz2 embryos at 52 hpf shows smaller livers in MO-wnt2 (J,J') and prt/wnt2bb embryos (K,K'), and an increasing reduction of the liver in MO-wnt2;prt/wnt2bb embryos (L-L'), which in more severe cases includes a reduction of 2F11-positive EHPD (d:M-N'); distribution of phenotypes indicated as percentages (n=65). (O-R') Tg(fabp10:dsRed)sz2 expression confirms the reduction of differentiating liver tissue in prt/wnt2bb embryos (P,P') and MO-wnt2 (Q,Q') and absence in MO-wnt2;prt/wnt2bb embryos (R,R') at 74 hpf, indicating that the liver defect fails to recover; Tg(XlEef1a1:GFP)sz2 and Tg(ela3l:EGFP)sz2 expression indicates the digestive tract and exocrine pancreas remain unaffected (O-R'). (A-H) Dorsal views and (I-R') ventral views of projections of confocal stacks; all anterior towards the top. gb, gall bladder; L, liver; p, pancreas.
Instructive activity of Wnt2 and Wnt2bb reveals hepatogenic potential of prospective non-hepatic endoderm

Wnt2 and Wnt2bb cooperative activity is essential for hepatoblast specification and growth, raising the possibility that both signals are sufficient. To test this, we examined overexpression of wnt2bb and wnt2 in the context of early liver development. To allow for temporal control as well as tracking of the wnt2bb-expressing cells, we generated a hsp70:mCherry-2A-wnt2bb construct, with a viral 2A linker peptide (Holst et al., 2006) for independent production of mCherry and Wnt2bb (see Fig. S3A in the supplementary material). First, we tested the activity of this construct by overexpressing wnt2bb around the time of hepatic specification at 18 hpf in prt/wnt2bb embryos. A significant rescue of liver size was observed in mutant embryos, as shown by cp expression at 50 hpf (40%, n=20) and Prox1 expression at 34 and 48 hpf (50%, n=8, and 55%, n=11, respectively; see Fig. S3B in the supplementary material), indicating that this construct produces active Wnt2bb. Interestingly, wnt2 overexpression at 18 hpf in prt/wnt2bb embryos, using the same experimental strategy, resulted in a significant increase of liver size at 48 hpf, confirming that wnt2 can compensate for lack of wnt2bb function, in agreement with our loss-of-function data (61%, n=13; data not shown). Conversely, transgenic overexpression of wnt2bb at 26 hpf can rescue liver size defects observed in Wnt2-depleted embryos when assayed for cp expression (78%, n=9; data not shown).

To examine the effect of excess wnt2bb or wnt2 on liver development, liver formation was analysed in embryos, in which ectopic expression was activated at 18 hpf, the time endogenous wnt2bb expression can be observed (Ober et al., 2006). Overexpression of either ligand led to a striking increase of the Prox1 domain (Fig. 3A-D'; see Fig. S4A-F' in the supplementary material). Therefore, we carried out BrdU-incorporation assays and examined cell proliferation by co-staining for BrdU and Prox1 at 26 hpf. Excess wnt2bb or wnt2 resulted in a twofold increase in hepatoblast proliferation (see Fig. S3E-H' in the supplementary material), supporting the finding that both ligands are required to promote hepatoblast proliferation. Candidate mediators of this proliferative activity are CyclinD1 and/or Myca, known targets of canonical Wnt signalling. qPCR analysis for both genes was performed 4 hours after activation of wnt2bb or wnt2 expression at 26 hpf and showed an increase of gene expression (see Fig. S3J in the supplementary material). Specifically, excess Wnt2bb led to a significant increase of cyclinD1 and myca expression compared with sibling controls (58% and 14%, respectively), indicating that both factors probably mediate Wnt2bb and possibly also Wnt2 signalling in cell proliferation.

To assess whether a defect in endoderm patterning also contributes to the observed liver enlargement, we examined the early hepatoblast domain by Prox1 expression. Overexpression of wnt2bb in Tg(XI-Eef1a1:GFP)S854 embryos resulted in a striking posterior expansion of Prox1 expression in embryos at 28 and 48 hpf (Fig. 3A-D'). The number of endodermal Prox1 cells was increased by 3.4 and 2.1-fold, respectively (Table 2). In wild type, Prox1-positive hepatoblasts are found at 28 hpf in a well-defined domain anterior to the endocrine pancreas (Fig. 3A), whereas in 52% of embryos with excess wnt2bb this domain is strongly enlarged, extending posteriorly beyond the pancreatic islet (Fig. 3B,B'; Table 2). This posterior expansion of Prox1-positive tissue was maintained at later stages and observed in 69% of injected embryos at 48 hpf (Fig. 3C-D'; Table 2). These data suggest that
Wnt2bb is sufficient to specify hepatoblasts within ectopic domains of the endoderm. At 48 hpf, Prox1 was expressed in hepatic and pancreatic exocrine cells, so we examined hepatic and pancreatic gene expression to confirm their fate. Posteriorly expanded cp expression at 44 hpf and Tg(fabp10:dsRed)$^{gb4}$ expression at 74 hpf revealed that these ectopic Prox1 cells differentiated into hepatocytes (Fig. 3E-H; Table 2). This liver increase was accompanied by a strong reduction or absence of exocrine pancreatic tissue, as visualised by Tg(ptf1a:eGFP)$^{jh1}$ and Tg(ela3l:GFP)$^{gz2}$ (Fig. 3C,D,G,H; Table 2). Consistently, prt/wnt2bb mutants exhibit an enlarged ventral pancreas at 50 hpf (Fig. 2I,K; E.A.O. and D. Y. R. Stainier, unpublished observation). Furthermore, pdx1 expression, which is found in the pancreas, extrapancreatic duct and anterior domains of the intestine, was reduced in embryos with ectopic wnt2bb (Fig. 4I,J). Although pancreatic and most of the intestinal expression seemed to be absent in these embryos, expression in the domain of the extrapancreatic duct tissue persisted. As a subset of MO-wnt2;prt/wnt2bb embryos display EHPD defects, we examined EHPD formation in embryos with transiently increased wnt2bb. In these embryos, the domain of strong 2F11 expression appeared dysmorphic and enlarged (Fig. 4K,L), which is in agreement with the remaining pdx1 expression in this domain. This suggests that ectopic Wnt2bb can convert not only prospective pancreatic exocrine, but also anterior intestinal tissue into liver and possibly EHPD.

Notably, ectopic Prox1 expression was observed in domains far more posterior than the endogenous domain from which the liver originates (Fig. 3A-D), with ectopic Prox1-positive cells predominantly located ventrally in the alimentary canal (Fig. 4A-H'). This suggests that while endoderm outside the normal hepatoblast domain is competent to respond to liver promoting Wnt signals, this ectopic competence is restricted to only ventral endoderm located posteriorly to the endogenous region.

### Table 2. Quantification of wnt2bb overexpression phenotypes

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<thead>
<tr>
<th>Time of HS: analysis</th>
<th>Number of controls</th>
<th>Number of injected embryos</th>
<th>Percentage of injected embryos with a posterior liver expansion</th>
<th>Fold increase of Prox1-positive hepatoblasts</th>
<th>P-value for each experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS 18 hpf: 28 hpf</td>
<td>23</td>
<td>27</td>
<td>51.85</td>
<td>2.64</td>
<td>2.5×10^{-10}</td>
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<tr>
<td>HS 18 hpf: 48 hpf</td>
<td>67</td>
<td>191</td>
<td>69.40</td>
<td>2.05</td>
<td>&lt;0.01</td>
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<tr>
<td>HS 18 hpf: 74 hpf</td>
<td>17</td>
<td>25</td>
<td>75</td>
<td>1.95</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HS 18 hpf: 48 hpf</td>
<td>18</td>
<td>43</td>
<td>90.57</td>
<td>0.24</td>
<td>&lt;1×10^{-15}</td>
</tr>
</tbody>
</table>

Average number of hepatic Prox1 cells or average volume of pancreatic Tg(ptf1a:eGFP)$^{jh1}$ expression was determined for each sample using Volocity software. Differences in liver or pancreas size are shown as fold-change between control and experimental embryos displaying a posterior liver expansion.

HS, heat shock.

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Fig. 4. Ventral digestive tract endoderm posterior to the hepatic domain is competent to respond to hepatogenic signals in vivo. (A-H') transient wnt2bb-overexpression in Tg(XlEef1a1:GFP)$^{gb4}$ embryos at 18 hpf leads to an expansion of the liver in posterior ventral tissues visualised by Prox1 staining at 28 hpf (A-D') and 48 hpf (E-H'). (I,J) pdx1 expression is significantly reduced in the pancreas and anterior intestinal domains (bracket) following wnt2bb-overexpression. (L) Embryos with excess wnt2bb exhibit ectopic Prox1 and strong 2F11 staining in the EHPD domain, revealing ectopic structures (arrows), compared with controls (K). (A,C,G,H) dorsal views (B,D,F,H',K,L) ventral views of projections of confocal stacks; (I,J) dorsal views; all anterior towards the top.
In summary, these data suggest that ectopic expression of \textit{wnt2bb} or \textit{wnt2} is sufficient to direct multipotent ventral digestive tract posterior to the endogenous hepatic domain to adopt a liver fate at the expense of pancreatic and anterior intestinal tissue, indicating that \textit{wnt2} and \textit{wnt2bb} expression requires tight spatiotemporal regulation to ensure formation of both liver and pancreas.

**\textit{wnt2} and \textit{wnt2bb} interact with \textit{frizzled5} in liver formation**

In embryos with excess \textit{wnt2} or \textit{wnt2bb}, the liver progenitor domain is posteriorly expanded at the expense of ventral pancreatic and anterior intestinal fates, raising the possibility that the wider competence of the digestive tract endoderm is mediated by a receptor of Wnt/\textit{β}-catenin signalling expressed in these domains. Knocking out the frizzled homolog 5 gene in mouse causes placental angiogenesis and yolk sac defects (Ishikawa et al., 2001), resembling those observed in \textit{Wnt2}-deficient embryos (Monkley et al., 1996). In zebrafish, \textit{fzd5} is expressed in the foregut and intestinal endoderm at the time of hepatoblast specification (see Fig. S5 in the supplementary material). Intriguingly, \textit{fzd5} appears to be expressed in the presumptive hepatic endoderm: in bilateral domains at 20 hpf, and in a midline position at 22 and 26 hpf. Fate-mapping studies of this domain at 14 hpf revealed that hepatoblast precursors are located lateral to the midline, compared with medially placed pancreas and digestive tract precursors (Chung et al., 2008), supporting the idea that \textit{fzd5} is expressed in liver precursors at the time of hepatoblast specification. In endoderm-less \textit{casanova}/\textit{sox32} mutant embryos (Alexander et al., 1999), this expression domain is absent, confirming its endodermal nature (data not shown). To test the possibility that \textit{fzd5} interacts with \textit{wnt2} and \textit{wnt2bb} in liver formation, a previously published MO (Cavodeassi et al., 2005) was used to deplete \textit{Fzd5} function. Lack of \textit{Fzd5} resulted in 82% of embryos with no, or a strongly reduced, domain of hepatic \textit{hhex} expression at 25 hpf (n=56; Fig. 5A,C). This phenotype was less severe at 28 hpf, with 88% of \textit{MO-fzd5} embryos showing a variably reduced liver progenitor domain (n=68; Fig. 5E,G). This is reminiscent of the \textit{prt/wnt2bb} mutant phenotype (Fig. 5B,F) (Ober et al., 2006) and suggests that \textit{fzd5} is required for early hepatoblast development. As the phenotype is not fully penetrant, other factors in addition to \textit{fzd5} may interact with \textit{wnt2} and \textit{wnt2bb} in early liver formation; or, alternatively, the \textit{Fzd5} knockdown is incomplete. To confirm that \textit{wnt2} and/or \textit{wnt2bb} interact genetically with \textit{fzd5}, we depleted \textit{Fzd5} in embryos lacking either \textit{Wnt2} or \textit{Wnt2bb}. These embryos exhibited a more severe reduction of hepatic \textit{hhex} expression at 28 hpf than either single knockdown, as the majority of \textit{MO-wnt2};\textit{MO-fzd5} and \textit{prt/wnt2bb};\textit{MO-fzd5} embryos displayed a complete loss of hepatoblasts (58%, n=24, and 68%, n=31, respectively) (Fig. 5E,H,I) and the remaining embryos showed a strong reduction. Consistent with these genetic interaction data, efficiency of either ligand to convert non-hepatic endoderm into liver fate was strongly decreased in the absence of \textit{Fzd5} function, as revealed by expression of \textit{hhex} at 28 hpf (Fig. 5J-L) and \textit{cp} at 48 hpf (Fig. 5M-R, data not shown). Excess of \textit{wnt2bb} in \textit{Fzd5}-depleted embryos resulted in only 28% of \textit{MO-fzd5} embryos (n=83) displaying an increased liver domain, in contrast to 85% of controls (n=88; Fig. 5M). Similar effects have been observed upon overexpressing \textit{wnt2} in \textit{MO-fzd5} (see Fig. S4I-K in the supplementary material). This suggests that \textit{fzd5} is required for early liver formation and provides the digestive tract endoderm with the competence to adopt a hepatic fate by mediating Wnt2 and Wnt2bb signalling.

**Wnt2 and Wnt2bb cooperate in swim bladder formation**

\textit{wnt2} and \textit{wnt2bb} expression in the LPM is at later stages not only adjacent to the forming liver, but also in close proximity to the prospective swim bladder (Fig. 1C), suggesting additional functions for both ligands in the formation of other foregut-derived organs. The swim bladder, which is thought to be structurally homologous to the lung and to carry out derived functions in teleosts, arises slightly anterior to the liver at 36 hpf (Winata et al., 2009). Examination of \textit{foxa1} and anterior gradient2 (\textit{agr2}) expression in the forming swim bladder (Fig. 6A,F), showed a loss of swim bladder expression in the majority of \textit{MO-wnt2};\textit{prt/wnt2bb} embryos (84%, n=117; Fig. 6D,I) and variable reduction in \textit{wnt2} and \textit{prt/wnt2bb} single loss-of-function embryos (Fig. 6B,C,G,H; see Fig. S1E in the supplementary material). Analysis of cleaved Caspase3 expression revealed that cell death does not contribute to swim bladder hypoplasia in \textit{prt/wnt2bb} and \textit{MO-wnt2} embryos at 42 hpf (data not shown). By contrast, BrdU incorporation assays carried out at the same stage showed a 47% reduction of swim bladder proliferation in \textit{prt/wnt2bb} embryos (n=14) and no consistent change in \textit{MO-wnt2} (n=13; Fig. 6I). These data suggest that combined Wnt2bb and Wnt2 activity is required for swim bladder formation, for specification and probably for proliferation. Intriguingly, excess Wnt2bb or Wnt2 supplied at different stages between 18 to 30 hpf does not alter swim bladder formation as assessed by \textit{foxa1} and \textit{agr2} expression at 48 hpf (see Fig. S4L-N in the supplementary material; data not shown), suggesting that both ligands are essential, but not sufficient, for swim bladder formation. Furthermore, examining \textit{foxa1} expression in \textit{MO-fzd5} embryos revealed in 55% of embryos a reduction of the swim bladder (n=73; Fig. 6E) similar to the one observed in \textit{MO-wnt2} or \textit{prt/wnt2bb} embryos, with a small subset showing swim bladder agenesis. These data indicate a crucial role for \textit{wnt2}, \textit{wnt2bb} and \textit{fzd5} in swim bladder formation, identifying essential factors of the poorly understood molecular network governing its development.

**DISCUSSION**

This study establishes Wnt2 and Wnt2bb as key factors in foregut patterning and hepatoblast proliferation in zebrafish (Fig. 7). We demonstrate that subsequent to Wnt2bb promoting liver specification (Ober et al., 2006), Wnt2 and Wnt2bb are required for hepatoblast proliferation and swim bladder development. In light of our findings, the spatiotemporal expression of these two ligands appears to be essential for patterning a pool of multipotent progenitors. Furthermore, we provide first mechanistic insights by showing that endodermal Frizzled5 interacts with Wnt2bb and Wnt2 in hepatoblast formation.

**Sequential roles for Wnt2bb and Wnt2 in liver formation**

Canonical Wnt/\textit{β}-catenin signalling has been implicated in different phases of liver development (Goessling et al., 2008; Nejak-Bowen and Monga, 2008). Initially, Wnt/\textit{β}-catenin signalling needs to be repressed for establishing the competence of the foregut tissue to differentiate into hepatic and pancreatic precursors (Dessimonz et al., 2006; Goessling et al., 2008; McLin et al., 2007). By contrast, depletion of \textit{β}-catenin shortly after organ specification in mouse and chick results in liver hypoplasia due to reduced proliferation and increased apoptosis (Sukswawang et al., 2004; Tan et al., 2008). Conversely, loss of a single copy of \textit{Apc}, a negative regulator of the pathway, results in elevated Wnt/\textit{β}-catenin signalling and
proliferation in the differentiating liver in zebrafish (Goessling et al., 2008). However, the identity of the secreted Wnt ligands regulating these processes remains largely unknown. In this study, we use both loss- and gain-of-function experiments to demonstrate that Wnt2 and Wnt2bb levels control liver size. We propose that both ligands act sequentially in hepatoblast specification and proliferation, exerting specific as well as overlapping functions. First, wnt2bb is required to specify the appropriate number of hepatoblasts in a timely manner from the multipotent endoderm (Ober et al., 2006). Although wnt2bb itself is not essential for liver induction, we demonstrate that wnt2, the expression of which is normally initiated only after hepatoblasts have been specified, can compensate for the loss of Wnt2bb in this process. Importantly, unlike in prt/wnt2bb mutants, the liver specification defect in embryos depleted of Wnt2bb and Wnt2 function fails to recover at least for several days, suggesting that the combined activity of these two ligands is essential for liver specification. In a second step, we identify Wnt2 and Wnt2bb as the endogenous ligands mediating early hepatoblast proliferation in zebrafish.

Intriguingly, Wnt/β-catenin signalling has been implicated in the regenerative response of the liver in rat and zebrafish (Goessling et al., 2008; Nejak-Bowen and Monga, 2008), emphasising the need to further understand the sequential requirements of Wnt signalling in hepatogenesis.
Liver specification – an interplay between broad endodermal competence and restricted hepatogenic signals

The precise spatiotemporal expression of both ligands in the LPM implicated them as important determinants for patterning the adjacent multipotent foregut endoderm. This notion is supported by our finding that overexpression of \textit{wnt2bb} and \textit{wnt2} at the time of hepatic specification led to ectopic posterior liver differentiation, which was accompanied by a severe depletion of ventral pancreatic tissue. This is consistent with recent findings reporting that \textit{wnt8} overexpression in zebrafish (Goessling et al., 2008) and stabilisation of β-catenin in prospective pancreatic tissue in the mouse (Heiser et al., 2006) lead to severe pancreas hypoplasia. Consistently, we observe an increase of pancreatic tissue in \textit{prt/wnt2bb} and \textit{MO-wnt2;prt/wnt2bb} embryos. It is conceivable that the smaller increase in \textit{MO-wnt2;prt/wnt2bb} than in \textit{prt/wnt2bb} embryos might be due to a function of Wnt signalling in pancreas proliferation, which in \textit{prt/wnt2bb} embryos is compensated for by Wnt2. This scenario is consistent with a study in mouse showing that early activation of β-catenin signalling leads to pancreas hypoplasia, whereas later activation promotes pancreas proliferation (Heiser et al., 2006). Altogether, these findings indicate that Wnt2bb and Wnt2 can directly or indirectly inhibit ventral pancreas formation and that precise expression levels of these Wnt ligands in the LPM are important to maintain a balance between liver and pancreas progenitors. Furthermore, our data support the concept that bipotential hepatopancreatic precursors reside in the ventral foregut endoderm (Chung et al., 2008; Deutsch et al., 2001). Tissue explant studies in mouse revealed that nascent foregut endoderm in culture exhibits default pancreatic fate as indicated by \textit{Pdx1} expression, whereas addition of Fgf to these cultures promotes the differentiation into \textit{Albumin}-positive hepatic tissue (Deutsch et al., 2001). These experiments suggested that foregut explants have the potential to differentiate into either liver or pancreas. Moreover, recent work in zebrafish provided in vivo data for bipotential hepatopancreatic precursors, showing that overexpression of Bmp2b can convert cells fated to become pancreas and alimentary canal into liver (Chung et al., 2008). This fate change is accompanied by repression of \textit{pdx1} and an increase of \textit{hhex} expression. The time window for this ability of Bmp2b precedes the effects we observe with Wnt2bb and Wnt2. Hence, it is possible that Bmp signalling acts permissively to facilitate liver proliferation.
formation by subsequent Wnt signalling. Alternatively, both pathways could control hepatic and pancreatic gene expression independently.

Our results further indicate that the endoderm posterior to the endogenous hepatic domain is competent to adopt a hepatic fate, and Wnt2bb and Wnt2 represent instructive rather than permissive signals. Notably, ectopic stabilisation of β-catenin in the mouse results in a similar posterior expansion of lung progenitors, at the expense of stomach tissue, but these progenitors fail to differentiate (Goss et al., 2009; Harris-Johnson et al., 2009). By contrast, the ectopic liver progenitors that form upon wnt2bb and wnt2 overexpression differentiate and express hepatocyte markers, suggesting a qualitative difference in the activation of the organotypic differentiation programmes. The observation that upon ectopic expression of wnt2 or wnt2bb hepatic tissue is induced in domains posterior to the foregut provides evidence that this part of the endoderm is competent to respond to hepatogenic signals. Notably, Vhnf1, FoxA and Gata factors, which have been shown to provide hepatic competence (Lokmane et al., 2008; Zaret et al., 2008), are expressed in posterior, non-liver endoderm around the time of liver specification. We show that fzd5, which is widely expressed in the digestive tract endoderm, not only interacts with wnt2bb and wnt2 in hepatoblast formation, but also mediates their ectopic activity posterior to the endogenous domain of prospective hepatoblasts. It will be interesting to uncover the mechanism preventing the formation of ectopic liver tissue in the anterior endoderm.

In embryos with posteriorly expanded Prox1 expression, liver gene expression is activated predominantly in cells located ventrally within the alimentary canal, suggesting a lack of competence in dorsal tissue. However, isolated dorsal intestinal endoderm from mouse or chick embryos can initiate liver gene expression in culture (Gualdi et al., 1996; Le Douarin, 1975), probably because these cells were removed from a repressive activity positioned in the dorsal midline. Our results demonstrate in vivo the potency of the nascent digestive tract endoderm to respond to hepatogenic signals and that dorsal tissue fails to respond, possibly owing to extrinsic repressive factors.

In summary, based on our results a model by which the expression of a receptor provides wider competence, while the spatially restricted ligands ensure hepatoblast specification only in a subset of competent cells. Hence, our findings strongly support a ‘code-like’ activity of Wnt signalling in foregut patterning and differentiation.

Unique and conserved functions of Wnt2 and Wnt2bb(b) in endoderm organogenesis

Intriguingly, recent work in mouse showed an essential role for β-catenin signalling and specifically for Wnt2 and Wnt2b in specification and proliferation of the lung, the equivalent of the zebrafish swim bladder (Goss et al., 2009; Harris-Johnson et al., 2009). These findings suggest a conserved function for Wnt2 and Wnt2bb(b) in swim bladder/lung formation between zebrafish and mouse. Their function in liver development appears to differ, as no gross hepatic defects have been described in mouse (Goss et al., 2009). These phenotypic differences might be explained by a lesser influence and/or different timely requirement of these ligands in hepatic development, or that other Wnt ligands fulfil the same role in mouse. Alternatively, anatomical characteristics of the digestive systems in the two species could provide an explanation. Zebrafish are stomach-less, and liver and swim bladder precursors are located in close proximity, whereas in mice, along with changes in digestive requirements, a stomach differentiates, separating the precursors of both organs (Harder, 1975; Serls et al., 2005). This suggests the interesting possibility that in mouse the source of the Wnt2 and Wnt2b signal is located next to the forming lung and is separated from the liver anlage by the forming stomach, leading to a different developmental requirement of both ligands in endodermal organogenesis. In X. laevis, Wnt2b has been implicated in stomach proliferation (Damianitsch et al., 2009). It will thus be interesting to determine Wnt2 and Wnt2b requirements in other vertebrates to better understand possible evolutionary changes in function. Comparing the molecular programmes controlling digestive system formation in different species promises to further our understanding as to how environmental fluctuations have contributed to altered functions of specific factors.

We demonstrate that anteroposteriorly restricted expression of different components of the Wnt signalling pathway is required for early steps of foregut-derived organogenesis, supporting the general concept of a Wnt ‘code’ playing an essential role in the development of the digestive system in vertebrates (Lickert et al., 2001; McBride et al., 2003; Theodosiou and Tabin, 2003). Dissecting the mechanisms that control the precise spatial and temporal expression of essential Wnt pathway components, such as wnt2bb, wnt2 and fzd5, as well as uncovering interactions with other pathways, will be crucial for unravelling the genetic network underlying the formation of each single organ.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


