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Specification of hepatopancreas progenitors in zebrafish by *hnf1ba* and *wnt2bb*

Joseph J. Lancman[1,*], Natasha Zvenigorodsky[2], Keith P. Gates[1], Danhua Zhang[1], Keely Solomon[3], Rohan K. Humphrey[4], Taiyi Kuo[2], Linda Setiawan[2], Heather Verkade[2,5], Young-In Chi[6], Ulupi S. Jhala[4], Christopher V. E. Wright[3], Didier Y. R. Stainier[2,‡,§] and P. Duc Si Dong[1,*§]

SUMMARY

Although the liver and ventral pancreas are thought to arise from a common multipotent progenitor pool, it is unclear whether these progenitors of the hepatopancreas system are specified by a common genetic mechanism. Efforts to determine the role of Hnf1b and Wnt signaling in this crucial process have been confounded by a combination of factors, including a narrow time frame for hepatopancreas specification, functional redundancy among Wnt ligands, and pleiotropic defects caused by either severe loss of Wnt signaling or Hnf1b function. Using a novel hypomorphic *hnf1ba* zebrafish mutant that exhibits pancreas hypoplasia, as observed in HNF1B monogenic diabetes, we show that *hnf1ba* plays essential roles in regulating β-cell number and pancreas specification, distinct from its function in regulating pancreas size and liver specification, respectively. By combining Hnf1ba partial loss of function with conditional loss of Wnt signaling, we uncover a crucial developmental window when these pathways synergize to specify the entire ventrally derived hepatopancreas progenitor population. Furthermore, our *in vivo* genetic studies demonstrate that *hnf1ba* generates a permissive domain for Wnt signaling activity in the foregut endoderm. Collectively, our findings provide a new model for HNF1B function, yield insight into pancreas and β-cell development, and suggest a new mechanism for hepatopancreatic specification.

KEY WORDS: Hnf1b, Pancreas, Liver, Wnt, Diabetes, MODY

INTRODUCTION

The liver and ventral pancreas, and presumably gall bladder and extra hepatopancreatic ducts, are believed to develop from a common multipotent progenitor population (hepatopancreas progenitors) present at early somitogenesis (Deutsch et al., 2001; Chung et al., 2008; Wandzioch and Zaret, 2009). In support of this model, multipotency of cells within this developing organ system appears to be maintained beyond early somitogenesis stages. For example, in *ptf1a* mutants, presumptive ventral pancreas progenitors will instead adopt intestinal (Kawaguchi et al., 2002), gall bladder, common bile duct (Burlison et al., 2008) or liver fate (Dong et al., 2008) (P.D.S.D., unpublished). Further observations supporting multipotency come from Sox17, Fgf10, and Hes1 loss-of-function studies where cells of the extra hepatopancreas ductal system can adopt pancreas or liver fate (Sumazaki et al., 2004; Fukuda et al., 2006; Dong et al., 2007; Spence et al., 2009). In addition, *fgf10* mutants develop ectopic pancreas cells in the liver (Dong et al., 2007), and both *fgf10* and *sox9* mutants develop liver cells in the pancreas (Dong et al., 2007; Seymour et al., 2012). These examples demonstrating that cells within the developing hepatopancreas organ system can readily switch fate is consistent with the liver, ventral pancreas, gall bladder and associated ducts arising from a common multipotent progenitor pool.

It remains unresolved whether a common genetic mechanism specifies this entire progenitor population. Although significant progress has been made in identifying factors necessary for either pancreas or liver specification, factors uniquely required for induction of the entire ventrally derived hepatopancreas system have yet to be discovered. Several transcription factors including Ptf1a and Pdx1 have been implicated in ventral pancreas specification, but neither factor is required for liver specification (Offield et al., 1996; Kawaguchi et al., 2002). In *Hhex* or *Gata4* mutants, the ventral pancreas is not specified, but early liver markers are expressed, suggesting liver specification has occurred (Bort et al., 2004; Bort et al., 2006; Watt et al., 2007). Although Foxa1 and Foxa2 function redundantly to establish endoderm competence necessary for liver specification (Kaestner, 2005; Lee et al., 2005), whether these factors act broadly to affect foregut endoderm patterning or have a more restricted role in liver and ventral pancreas specification has not been addressed .

Zebrafish and mouse embryos with severe or complete loss of HNF1B (also known as HNF1β, TCF2 and VHNF1) function exhibit profound foregut regionalization defects and subsequently fail to specify the liver and both the dorsal and ventral pancreas (Sun and Hopkins, 2001; Haumaitre et al., 2005; Lokmane et al., 2008). For this reason, it is unresolved whether the liver or pancreas agenesis defects observed in severe *hnf1b* loss-of-function mutants are secondary to the earlier foregut patterning defects. Loss of *wnt2bb* in zebrafish can lead to a low penetrant liver specification defect. Although the penetrance of this defect is increased when

[1]Sanford Children's Health Research Center, Programs in Genetic Disease, Development and Aging, and Stem Cell and Regenerative Biology, Graduate School of Biomedical Sciences, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA. [2]Department of Biochemistry and Biophysics, Programs in Developmental Biology, Genetics and Human Genetics, and the Diabetes Center and Liver Center, University of California, San Francisco, 1550 Fourth Street, San Francisco, CA 94158, USA. [3]Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA. [4]Pediatric Diabetes Research Center, UCSD School of Medicine, La Jolla CA 92037, USA. [5]School of Biological Sciences, Monash University, Clayton, VIC 3800, Australia. [6]Hormel Institute, University of Minnesota, Austin, MN 55912, USA.

*These authors contributed equally to this work.
‡Present address: Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany
§Authors for correspondence (didier.stainier@mpi-bn.mpg.de; ducdong@sanfordburnham.org)

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combined with *wnt2* loss of function, swimbladder agenesis is also observed and, importantly, ventral pancreas specification still occurs (Ober et al., 2006; Poulain and Ober, 2011). It is not known whether other Wnt ligands act redundantly with *wnt2bb* and *wnt2* to specify the ventral pancreas. Nevertheless, Wnt signaling has been implicated in pancreas development. Studies in frog showed that Wnt signaling before early somitogenesis plays a negative role in liver and pancreas development by inhibiting foregut identity (McLin et al., 2007). Studies in mice suggest that ectopic Wnt signaling during early pancreas development can antagonize pancreas specification (Heller et al., 2002; Heiser et al., 2006; Murtaugh, 2008). However, between these developmental stages, whether endogenous Wnt signaling regulates ventral pancreas specification has yet to be determined.

HNF1B is the gene implicated in maturity onset diabetes of the young 5 (MODY5), one of ten monogenic, dominantly inherited forms of early-onset diabetes (Horikawa et al., 1997; Borowiec et al., 2009; Nyunt et al., 2009; Wang et al., 2009). Based on genome-wide association (GWA) studies, *HNF1B* and its closely related paralog *HNF1A* (*MODY3*), are also associated with type 2 diabetes (T2D), suggesting broader significance for studying these monogenic diabetes genes (Frayling, 2007; Wang et al., 2009; Voight et al., 2010). Loss of insulin producing pancreatic β-cells is characteristic of nearly all forms of diabetes and is the underlying factor for diabetic insulin dependence. While it has been suggested that HNF1B is important for liver insulin sensitivity, it remains unclear whether its dysfunction in the pancreas, particularly the β-cells, contributes to the etiology of *HNF1B*-related diabetes (Brackenridge et al., 2006; Kornfeld et al., 2013). Although targeted knockout of *Hnf1b* from β-cells in mice leads to impaired glucose tolerance, fed or fasted plasma glucose and insulin levels were not affected, suggesting that Hnf1b may not be crucial for maintaining β-cell function (Wang et al., 2004). However, it is not known whether Hnf1b is required for normal β-cell development. In support of a role in β-cell development, lineage-tracing studies in mice reveal that Hnf1b is expressed in pancreas endocrine progenitors that can give rise to β-cells during development (Solar et al., 2009). GWA studies have also implicated the Wnt pathway, including *WNT2B*, as the highest ranked pathway associated with T2D (Perry et al., 2009). By analyzing the role of *wnt2bb* and *hnf1b* in pancreas development, we aim to advance our understanding of β-cell development and diabetes etiology.

The heterozygous nature of the *HNF1B* mutations in individuals with MODY5 suggests that partial loss of HNF1B function can lead to disease pathologies. However, to date only complete or severe loss-of-function *Hnf1b* alleles have been examined in mouse and zebrafish (Barbacci et al., 1999; Sun and Hopkins, 2001; Haumaitre et al., 2005; Lokmane et al., 2008). As would be expected, *Hnf1b*-null mutants exhibit more pleiotropic and severe defects than those observed in individuals with MODY5, limiting their utility as development and disease models. Whether HNF1B plays a distinct role in β-cell development or in pancreas and liver specification has been difficult to study because of the broader endoderm patterning defects associated with its severe loss of function. Our studies provide new insights into HNF1B function by uncoupling these defects. We report here a novel hypomorphic *hnf1ba* zebrafish mutant that does not exhibit significant foregut endoderm regionalization defects. Our analysis of this mutant leads to several important advances. We find that partial loss of *hnf1ba* function in zebrafish can cause MODY5-like pancreas defects. We also find that normal levels of *hnf1ba* function are distinctly required for proper β-cell numbers and for pancreas specification. Furthermore, from a synergistic genetic interaction between *hnf1ba* and *wnt2bb*, we uncover a new mechanism for hepatopancreas progenitor specification, revealing that a common genetic program, within a narrow developmental time window, specifies progenitors of the liver, ventral pancreas, gall bladder and associated ducts.

MATERIALS AND METHODS

Zebrafish strains

Zebrafish were raised and maintained under standard laboratory conditions. We used the following mutant and transgenic lines: *liger*^{s430} (*hnf1ba*^{s430}), *hnf1ba*^{hi2169} (Sun and Hopkins, 2001), *wnt2bb*^{s404} (Ober et al., 2006), *Tg(hsp70l:wnt8-GFP)*^{w34} (Weidinger et al., 2005), *Tg(elastase:GFP);Tg(lfabp:dsRed)*^{gz2} (Wan et al., 2006), *Tg(hsp70l:dkk1-GFP)*^{w32} (Stoick-Cooper et al., 2007) and *Tg(ptf1a:eGFP)*^{jh1} (Godinho et al., 2005).

Genotyping of mutant embryos

hnf1ba^{s430} genotyping

Genomic DNA was amplified with (F:5′-AGCAGCACAATATCCC-TCAGCGCGAGGTCG-3′) and (R:5′-AAAACCGTCACATGCAACAA-3′) primers. Amplicons were then digested overnight with Taq$^{\alpha}$1 (NEB). The forward primer introduces a mismatch creating a Taq$^{\alpha}$1 restriction site in the *hnf1ba*^{s430} amplicon (Neff et al., 1998). Following digestion, the wild-type allele generates a band of 195 bp and the *liger*^{s430} allele a band of 165 bp.

wnt2bb^{s404} genotyping

Genomic DNA was amplified with (F:5′-CGTTCGTATACGCGATCTCC-3′) and (R:5′-TTCCACAGCGGTTGTTATGA-3′) primers followed by digestion with Fok1. The wild-type allele generates a band of 258 bp and the *wnt2bb*^{s404} allele a band of 152 bp. *hnf1ba*^{hi2169} mutants were genotyped as described previously (Sun and Hopkins, 2001).

Morpholino injections

One-cell stage embryos were injected with varying amounts of a *hnf1ba* morpholino (0.28-1.7 ng) (5′-GGGAAATGCGGTATTGTGATCTTTC-3′) (Gene Tools).

Heat-shock conditions

Embryos were heat-shocked at 21 hpf by adding egg-water pre-warmed to 38°C for *Tg(hsp70l:dkk1-GFP)*^{w32} and 39°C for *Tg(hsp70l:wnt8-GFP)*^{w34} and maintained at 37°C and 38°C, respectively, for 7 hours. Following heat-shock, embryos were sorted, returned to 27.5°C and collected at specified stages.

Fluorescent immunohistochemistry

Whole-mount fluorescent immunohistochemistry was performed as previously described (Dong et al., 2007), using the following antibodies: mouse anti-Islet1/2 [1:10; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-pan-Cadherin (1:5000; Sigma), guinea pig anti-Insulin (1:500; Biomeda), mouse anti-Synaptic Vesicles (SV2; 1:20; DSHB), guinea pig anti-Pdx1 (1:200), rabbit anti-Prox-1 (1:200; Millipore) and mouse anti-Myosin to label somites following *in situ* hybridization (1:20; DSHB, F59), fluorescently conjugated Alexa secondary antibodies (Molecular Probes) and DAPI. Control and mutant (or morphant) embryos were stained in the same tube for each experiment. To analyze β-cell numbers, day 3 and 5 *hnf1b*^{s430} and wild-type embryos were processed for confocal imaging using DAPI and antibodies against insulin and synaptic vesicles. *Z*-stacks (0.8 μm steps) were collected for the entire islet with optical slices captured at a focal depth of 1.6 μm. Samples were imaged using a Zeiss LSM5 Pascal or Zeiss 710 confocal microscope.

Reporter constructs and assay

Full-length zebrafish wild-type and mutant *hnf1ba* (V147E) were subcloned into pcDNA3-Myc vector and co-transfected with β28-Luciferase reporter (a kind gift from Dr G. Crabtree, Stanford University, CA, USA) containing three copies of the HNF1-binding site from the fibrinogen promoter and with RSV-β-Gal, into Min6 or HepG2 cells in the absence or presence of both CMV Flag-pCAF or CMV Flag-CBP expression vectors, as indicated.

Luciferase was measured using a luminometer, and values normalized for expression of β-Gal as described.

In situ hybridization

In situ hybridization was performed as described with minor modifications (Yelon et al., 1999; Dong et al., 2007). Following *in situ* hybridization, immunohistochemistry was performed to fluorescently label somites as previously described (Dong et al., 2007; Huang et al., 2008). Images were captured using a Zeiss 710 confocal microscope with AxioPlan 4.8 software. *In situ* hybridization and immunofluorescent images were captured for a single sample and merged using Photoshop CS3. Probes for *hnf1ba*, *hhex*, *prox1*, *cmyc* (*myca* – Zebrafish Information Network) and *wnt2bb* were donated and are described elsewhere (Ho et al., 1999; Sun and Hopkins, 2001; Yamaguchi et al., 2005; Ober et al., 2006; Shin et al., 2011). Experimental and control embryos were stained in the same tube and all experiments were carried out at least twice using distinct clutches.

RESULTS

Identification and molecular characterization of a new *hnf1ba* hypomorphic zebrafish mutant

From a zebrafish ENU mutagenesis screen (Ober et al., 2006), we identified *liger*^{s430}, a recessive mutant that exhibits a variably smaller pancreas, as marked by *Tg(ptf1a:*eGFP*)*^{jh1} expression (Godinho et al., 2005; Dong et al., 2008) (Fig. 1A,B, arrows indicate pancreas). *ptf1a*, which encodes a basic helix-loop-helix transcription factor, is expressed in the pancreatic anlagen and is required for ventral pancreas specification (Kawaguchi et al., 2002; Lin et al., 2004; Zecchin et al., 2004; Dong et al., 2008). The *liger*^{s430} mutation is linked to the simple sequence length polymorphism (SSLP) marker z4396 on chromosome 15, in the same region as *hnf1ba* (zfin.org).

Complementation analysis with a severe *hnf1ba* mutant, *hnf1ba*^{hi2169}, yielded transheterozygotes that phenocopied the *liger*^{s430} homozygous variable pancreas hypoplasia (with greater severity and penetrance), indicating that the *liger*^{s430} mutation is a hypomorphic allele of *hnf1ba* (Fig. 1C-F). In addition, *liger*^{s430} homozygous and *liger*^{s430}/*hnf1ba*^{hi2169} transheterozygous embryos do not exhibit kidney cysts (not shown) or liver agenesis, as reported in the *hnf1ba*^{hi2169}, *hnf1ba*^{hi548}, *hnf1ba*^{hi1843}, *hnf1ba*^{wi408} and *hnf1ba*^{la550} homozygotes, providing evidence that *liger*^{s430} is hypomorphic to these published alleles (Sun and Hopkins, 2001; Wiellette and Sive, 2003; Song et al., 2007). Morpholino (MO) antisense knock-down of *hnf1ba* translation in embryos (morphants) phenocopies the range of pancreas hypoplasia and agenesis defects observed in *liger*^{s430} homozygotes and *liger*^{s430}/*hnf1ba*^{hi2169} transheterozygotes, depending on the amount injected (Fig. 1G-I; supplementary material Fig. S3), providing further evidence that the *liger*^{s430} mutation is a hypomorphic allele.

Sequence analysis of *hnf1ba* in *liger*^{s430} mutants revealed a transversion of a thymine to an adenine in the coding region at position 440, resulting in a valine to glutamic acid (V147E) missense mutation (Fig. 1J). This valine is highly conserved among vertebrates and is in the DNA-binding POU-specific domain where MODY5 and MODY3 (Hnf1a) missense mutations are most commonly found (Fig. 1K) (Chi et al., 2002; Gong et al., 2004; Edghill et al., 2006a). Based on crystal structure data of human HNF1B in complex with DNA, V140 in human HNF1B, which is homologous to V147 in zebrafish (Fig. 1K), is predicted to be involved in the formation of the POU-specific domain hydrophobic core but not make direct contact with DNA (supplementary material Fig. S1A) (Lu et al., 2007). Therefore, substitution of a highly charged group (V147E) is predicted to perturb the hydrophobic core and disrupt protein structure and function.

Consistent with the predicted structural model, we find that when compared with wild-type zebrafish Hnf1ba, V147E Hnf1ba fails to efficiently activate transcription of a luciferase reporter driven by a multimerized HNF1-binding element of the fibrinogen gene (supplementary material Fig. S1B) (Chi et al., 2002). In addition, although decreased relative to wild-type Hnf1ba, V147E Hnf1ba transcriptional potential can be enhanced by known co-activators pCAF and CBP (Barbacci et al., 2004), further supporting that V147E Hnf1ba retains some function (supplementary material Fig. S1B). Together with genetic linkage to the *hnf1ba* locus, phenocopy of *liger*^{s430} homozygous phenotypes by *hnf1ba* knock-down, failure of complementation by *hnf1ba*^{hi2169} and substitution of a highly conserved residue of Hnf1ba, we conclude that *liger*^{s430} (now designated *hnf1ba*^{s430}) is caused by a hypomorphic mutation in

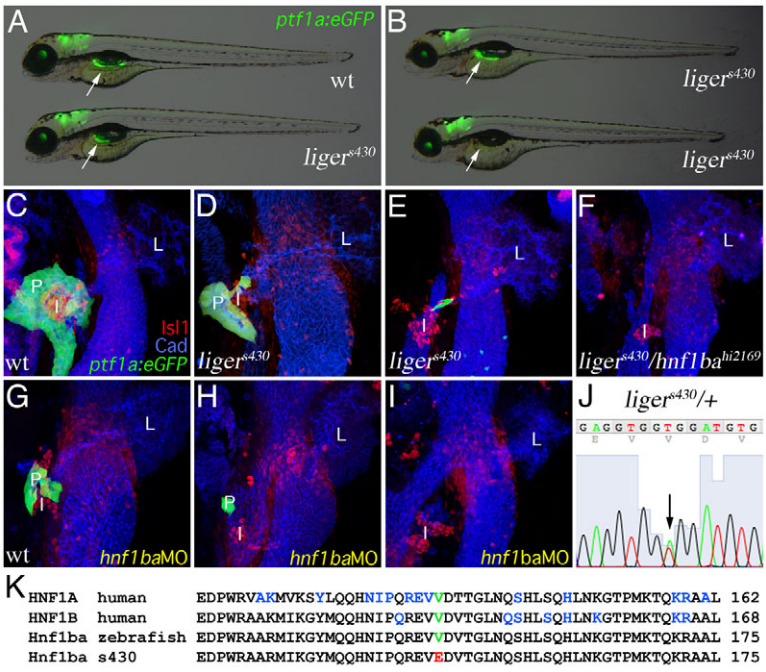

Fig. 1. *liger*^{s430} is a hypomorphic *hnf1ba* zebrafish mutant and exhibits MODY5-like pancreas hypoplasia. (**A**,**B**) Merged fluorescent/bright-field micrographs of 4 dpf *Tg(ptf1a:eGFP)*^{jh1} (arrows indicate pancreas) wild type (A, top) and *liger*^{s430} mutants showing the most common severity of pancreas hypoplasia (A, bottom). In *liger*^{s430} mutant embryos, the pancreas can be either nearly normal in size (B, top; <10%) or completely absent (B, bottom; <10%). (**C-I**) Three-dimensional rendering of the foregut endoderm of 80 hpf *Tg(ptf1a:eGFP)*^{jh1} wild-type (C), *liger*^{s430} (D,E), *liger*^{s430}/*hnf1ba*^{hi2169} (F) and hnf1ba-MO injected (G-I) embryos stained for Isl1 and cadherin demonstrating complementation failure by *hnf1ba*^{hi2169} (F) and phenocopy with Hnf1ba translational knock-down (compare D-F with G-I, respectively). (**J**) Genomic sequence from a *liger*^{s430} heterozygote indicating a molecular lesion (arrow; double peek) in *hnf1ba*. (**K**) Amino acid alignment covering part of the atypical POU-specific domain of human HNF1A and HNF1B and wild-type zebrafish and *liger*^{s430} Hnf1ba. Red font denotes the valine to glutamic acid substitution at position 147 in *liger*^{s430}. Blue font indicates amino acids affected by mis-sense mutations in MODY3 and MODY5.

hnf1ba. We note that *hnf1bb*, a paralog of *hnf1ba* in zebrafish, was not reported to be expressed in the developing foregut endoderm (Choe et al., 2008; Wingert and Davidson, 2011; Naylor et al., 2013). Because loss of *hnf1ba* in zebrafish and *Hnf1b* in mouse exhibit similar foregut endoderm phenotypes (Sun and Hopkins, 2001; Haumaitre et al., 2005; Lokmane et al., 2008), we suggest that *hnf1ba* is the primary functional ortholog of mammalian *Hnf1b* in foregut development.

hnf1ba^{s430} hypomorphs exhibit a MODY5-like pancreas hypoplasia and have reduced β-cell numbers

Foregut regionalization defects and pancreas agenesis in severe Hnf1b loss-of-function zebrafish and mouse mutants limit their utility as models for Hnf1b function in the developing and adult pancreas (Sun and Hopkins, 2001; Haumaitre et al., 2005). With this newly identified hypomorphic *hnf1ba* mutant, we have the unique opportunity to examine the consequence of Hnf1ba partial loss of function during pancreatogenesis. We find that the developing *hnf1ba*^{s430} mutant exocrine pancreas, which is thought to arise exclusively from the ventral pancreas in zebrafish (Field et al., 2003), is variably reduced in size based on *Tg(ptf1a:*eGFP*)*^{jh1} expression (Fig. 1A,B). In fewer than 15% of *hnf1ba*^{s430} homozygotes (n>300), the ventral pancreas can appear either normal in size (<10%) or completely absent (<10%), with variation between clutches. Because we find that MODY5-like pancreas hypoplasia can result from a quantitative loss of Hnf1ba (via translational knock-down), we propose that pancreas hypoplasia in MODY5 is due to decreased levels of HNF1B function. The dorsal pancreas, which comprises primarily endocrine cells in zebrafish (Field et al., 2003; Hesselson et al., 2009), is dysmorphic in *hnf1ba*^{s430} homozygotes. In mutant embryos, endocrine cells, which arise from both the dorsal and ventral pancreas, can fail to form a single islet cluster. Furthermore, the clusters of endocrine cells that do form do not organize into an islet with a central core of β-cells, normally observed in zebrafish and mice. We find a significant and consistent reduction in the number of β-cells in *hnf1ba*^{s430} homozygotes, regardless of exocrine size, although the severity of reduction generally correlates with exocrine size (Fig. 2A). To determine whether the roles of *hnf1ba* in regulating β-cell number and exocrine size are distinct, we compared β-cell numbers in 80 hpf wild-type embryos with those of 125 hpf mutants with a similar or larger exocrine pancreas. We find that age-matched or older mutant embryos with an exocrine pancreas similar to or larger than that of an 80 hpf wild type have significantly fewer β-cell numbers (n>20) (Fig. 2B-G; supplementary material Fig. S2A-D). This finding indicates that *hnf1ba* regulation of β-cell number is distinct from its regulation of pancreas size.

hnf1ba plays a distinct role in ventral pancreas specification

Although severe loss of *hnf1ba* does lead to ventral pancreas agenesis in zebrafish, it also causes loss of adjacent endodermal organs, including the liver, dorsal pancreas and swim bladder, indicating a more profound role for *hnf1ba* in posterior foregut patterning (Sun and Hopkins, 2001). In *Hnf1b*-null mice, in which ventral pancreas agenesis occurs, only rudiments of non-differentiating liver and dorsal pancreas buds were observed, also suggesting a broader role for *Hnf1b* in endoderm development and potentially obscuring a distinct role in pancreas specification (Haumaitre et al., 2005). Consistent with a role for Hnf1b in AP (anterior/posterior) patterning of the foregut endoderm, both

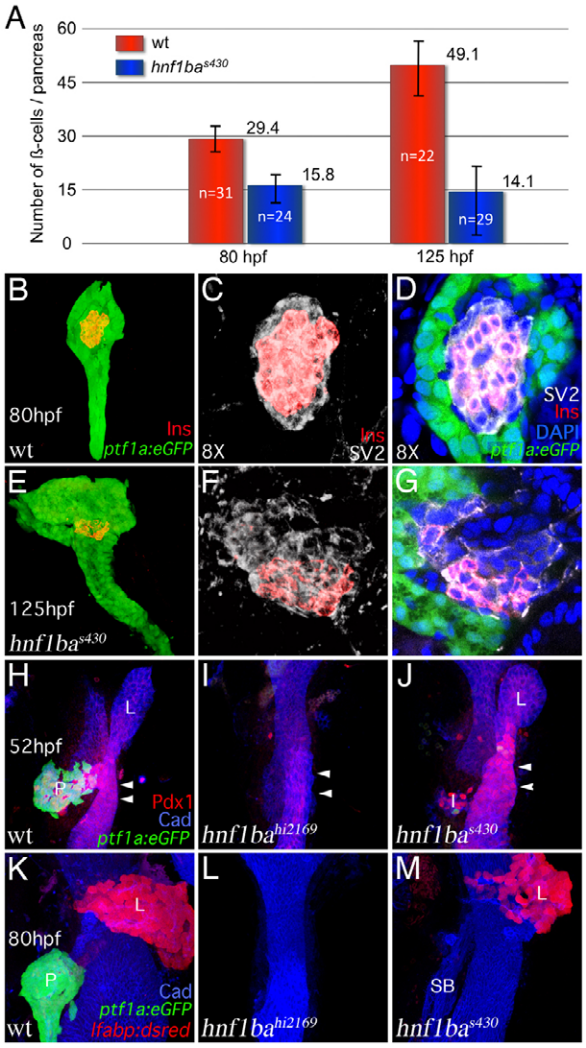

Fig. 2. Distinct roles for Hnf1ba in regulating β-cell numbers and ventral pancreas specification. (**A**) β-Cell nuclei numbers from 80 and 125 hpf wild-type and *hnf1ba*^{s430} mutants, indicating that at either stage, *hnf1ba*^{s430} mutants have significantly fewer β-cells. *hnf1ba*^{s430} mutants (125 hpf) have significantly fewer β-cells than 80 hpf wild-type embryos. Error bars represent s.d. (**B-G**) Fluorescent confocal microscopy of *Tg(ptf1a:eGFP)*^{jh1} 80 hpf wild type (B-D) and 125 hpf *hnf1ba*^{s430} mutant (E-G) pancreas stained for insulin antibodies, SV2 antibodies and DAPI to mark β-cells (red), endocrine cells (white) and nuclei (blue), respectively. Three-dimensional rendering of red and green channels showing an *hnf1ba*^{s430} mutant at 125 hpf with a larger pancreas (E) than wild-type (B). (C,F) Magnification (8×) of B and E (red and white channels only) to show mildly disorganized islet cells. (D,G) *Z*-focal plane of C and F (with all channels) demonstrating moderately reduced β-cell number in the *hnf1ba*^{s430} mutant islet. For a more severe example, see supplementary material Fig. S2. (**H-M**) Three-dimensional rendering of 52 hpf *Tg(ptf1a:eGFP)*^{jh1} (pancreas, green) foregut endoderm in wild-type (H), *hnf1ba*^{hi2169} mutants (I) and *hnf1ba*^{s430} embryos (J) stained for Pdx1 to mark the duodenal intestine and cadherin to mark the endoderm epithelium. In contrast to *hnf1ba*^{hi2169} (I), Pdx1 expression (arrowheads) in the intestine and dorsal pancreatic islet (I) is not lost in *hnf1ba*^{s430} embryos (J). (K-M) Three-dimensional rendering of 80 hpf *Tg(ptf1a:eGFP)*^{jh1}; *Tg(lfabp:dsRed)*^{gz2} (pancreas, green; liver, red) foregut endoderm in wild-type (K) *hnf1ba*^{hi2169} (L) and *hnf1ba*^{s430} embryos (M) stained for cadherin showing specific loss of ventral pancreas in *hnf1ba*^{s430} hypomorphic mutants. Unlike the *hnf1ba*^{hi2169} (L), liver (L) and swimbladder (SB) are not lost in the *hnf1ba*^{s430} embryos.

zebrafish and mouse *Hnf1b*-null mutants exhibit a posterior expansion of *hnf3b* and *sonic hedgehog* (*shh*) expression into the presumptive duodenum (posterior foregut), the region from which the ventral and dorsal pancreas and liver develop (Sun and Hopkins, 2001; Haumaitre et al., 2005). Hedgehog signaling in the anterior endoderm is required early to pattern the anterior foregut via repression of more posterior endoderm fate (duodenum/pancreas/liver) (Hebrok, 2003; Wan et al., 2006). Therefore, posterior expansion of *shh* expression would be expected to inhibit posterior foregut patterning events. Accordingly, zebrafish and mouse *hnf1b*-null embryos exhibit loss of expression of the posterior foregut endoderm (pancreas/duodenum) marker, *pdx1*, but not loss of the anterior foregut/endoderm markers *hnf3b*, *nkx2.1* and *irx2* (Sun and Hopkins, 2001; Haumaitre et al., 2005; Lokmane et al., 2008). These observations indicate a conserved effect of severe *hnf1b* loss-of-function mutations on the AP patterning of the vertebrate foregut (Sun and Hopkins, 2001; Haumaitre et al., 2005). In contrast to *hnf1ba*^{hi2169} mutants, AP foregut endoderm patterning appears normal in hypomorphic *hnf1ba*^{s430} embryos with pancreas agenesis. Duodenal Pdx1 expression is not lost in these *hnf1ba*^{s430} mutants, suggesting that the duodenal region (posterior foregut) is properly patterned (Fig. 2J). More important, adjacent endodermal organs (swim bladder, dorsal pancreas and liver) do develop and differentiate in *hnf1ba*^{s430} mutants with ventral pancreas agenesis, further indicating that foregut AP patterning is not significantly impaired (Fig. 2K-M). These results reveal that ventral pancreas development is more sensitive to reduced Hnf1ba function than neighboring tissues, thereby suggesting that *hnf1ba* is distinctly required for ventral pancreas specification, independent of its role in foregut endoderm regionalization and liver specification. Our *in vivo* data are consistent with previous molecular studies suggesting that Hnf1b transcriptionally activates Hnf6 to regulate Pdx1 and ventral pancreas specification (Poll et al., 2006).

Synergistic genetic interaction between *hnf1ba* and *wnt2bb* reveals their novel role in hepatopancreas specification

It is unresolved whether *hnf1b* also plays a distinct role in liver specification because of the difficulty in uncoupling the liver agenesis phenotype from the foregut regionalization defects exhibited by severe *hnf1b* mutants (Sun and Hopkins, 2001; Lokmane et al., 2008). Because *wnt2bb* mutants display rare liver specification (<1%, *n*=200) and transient hypoplasia defects (Ober et al., 2006) (Fig. 3A,B), and because *hnf1ba*^{s430} mutants exhibit variable but mild hepatic hypoplasia (Fig. 3A,C), we tested for a synergistic genetic interaction between *hnf1ba*^{s430} and *wnt2bb*^{s404} with respect to liver specification. Remarkably, about 80% of the *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants (44/55) completely lack hepatocytes, as assessed by *Tg(lfabp:dsRed)*^{gz2} expression, indicating that *hnf1ba* is indeed required for liver specification. The penetrance and severity of the liver agenesis and hypoplasia defects in double mutants is significantly greater than the additive

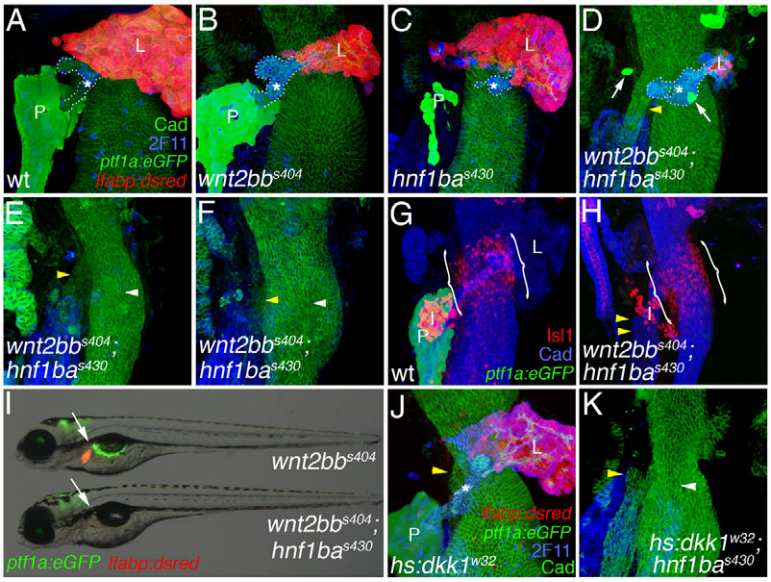

Fig. 3. *hnf1ba* and *wnt2bb* cooperate to specify the hepatopancreas organ system after mid-somitogenesis. (**A-F**) Three-dimensional rendering of 80 hpf *Tg(ptf1a:eGFP)*^{jh1}; *Tg(lfabp:dsRed)*^{gz2} (pancreas, green; liver, red) foregut endoderm in wild type (A), *wnt2bb*^{s404} mutant (B), *hnf1ba*^{s430} mutant (C) and *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants (D-F) stained for cadherin (epithelial endoderm) and with monoclonal antibody 2F11 to mark extra hepatopancreas ducts (*) (dashed outline). Relative to wild type, the *wnt2bb*^{s404} mutant liver (L) and the *hnf1ba*^{s430} mutant pancreas (P) are reduced in size. The *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutant liver and pancreas (arrow) are more severely reduced, or lost. Double mutants can exhibit complete loss of the hepatopancreas system (E,F; white arrowheads). (**G**,**H**) Three-dimensional rendering of 80 hpf *Tg(ptf1a:eGFP)*^{jh1} (pancreas, green) foregut endoderm in wild-type (G) and *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants (H) stained for cadherin and Isl1 to mark the hepatopancreas mesenchyme (brackets) and islet (I). Double mutants lacking the entire hepatopancreas system still develop Isl1+ mesenchyme and islet (yellow arrowheads indicate the swimbladder). (**I**) Merged fluorescent/brightfield micrographs of 4 dpf *Tg(ptf1a:eGFP)*^{jh1}; *Tg(lfabp:dsRed)*^{gz2} *wnt2bb*^{s404} mutant (top) and *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants (bottom) showing no obvious body phenotypes other than liver and pancreas agenesis (arrows). (**J**,**K**) Three-dimensional rendering of 80 hpf foregut endoderm, heat-shock-induced *Tg(hsp70l:dkk1-GFP)*^{w32}; *Tg(ptf1a:eGFP)*^{jh1}; *Tg(lfabp:dsRed)*^{gz2} and *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430}; *Tg(ptf1a:eGFP)*^{jh1}; *Tg(lfabp:dsRed)*^{gz2} stained for cadherin and with 2F11. Heat-shock-induced expression of *dkk1-GFP* in wild-type background does not lead to liver or pancreas agenesis (J), whereas in the *hnf1ba*^{s430} mutant background, the hepatopancreas system is completely lost (K) (white arrow). The swimbladder (yellow arrowhead) is not lost in *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants or in heat-shock-induced *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} mutants (D-F,H,K).

penetrance and severity of the single mutants, indicating a synergistic genetic interaction. Surprisingly, pancreatic agenesis/hypoplasia is also more severe and penetrant in double mutants (Fig. 3D), revealing a novel role for *wnt2bb* signaling in pancreas specification (*wnt2bb*^{s404} mutants do not exhibit obvious pancreas hypoplasia). Strikingly, in 6% of the double mutants (3/48), the entire ventrally derived hepatopancreas system, including the pancreas, gallbladder, extra-hepatopancreas ducts [assessed with 2F11 antibodies (Crosnier et al., 2005; Dong et al., 2007)] and liver, is absent (Fig. 3E,F), suggesting a more profound role for *hnf1ba* and *wnt2bb* in specifying these developmentally associated organs (supplementary material Table S1). Islet1 (Isl1) expression in mesenchymal cells that surround the extra-hepatopancreas ducts and adjacent intestine (Dong et al., 2007) appears normal in these double mutants (Fig. 3G,H). Importantly, neighboring endodermal organs, including the swim bladder and dorsal pancreas, do develop in these double mutants, indicating that foregut AP patterning is not significantly affected in the *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants. Other than hepatopancreas organ system defects, *wnt2bb*^{s404}; *hnf1ba*^{s430} double homozygous embryos are indistinguishable from their wild-type and single mutant siblings, based on brightfield microscopy (Fig. 3I; Fig. 1A,B). Therefore, agenesis defects observed in the *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants are highly restricted to ventrally derived hepatopancreas organs. Furthermore, unlike *wnt2bb*^{s404} or *hnf1ba*^{s430} single mutants, which can be homozygous viable as adults, double mutants do not survive beyond 9 dpf, indicating a synthetic lethal genetic interaction. To our knowledge, this is the first report of a genetic condition that leads to specific and complete agenesis of the entire ventrally derived hepatopancreas system, demonstrating that the liver and ventral pancreas are specified by a common genetic mechanism.

Wnt signaling interacts with *hnf1ba* after mid-somitogenesis to specify hepatopancreas progenitors

Current models suggest that genetic events regulating liver and pancreas specification occur during early somitogenesis (Chung et al., 2008; Wandzioch and Zaret, 2009; Shin et al., 2011). However, the liver and ventral pancreas do not bud until after mid-somitogenesis, suggesting that other genetic events may more directly regulate hepatopancreas fate in the foregut endoderm. To assess the temporal requirement of the synergistic genetic interaction between Wnt signaling and Hnf1ba in hepatopancreas specification, we mis-expressed the Wnt/β-catenin antagonist Dickkopf (Dkk1) (Glinka et al., 1998) in a temporally specific manner via heat-shock using the *Tg(hsp70l:dkk1-GFP)*^{w32} line in the *hnf1ba*^{s430} background (Fig. 3J,K). Heat-shock of *Tg(hsp70l:dkk1-GFP)*^{w32} or *hnf1ba*^{s430} embryos for 7 hours beginning at 21 hours post fertilization (hpf, ~24-somite stage, 3 hours before initial expression of the hepatopancreas progenitor markers *hhex* and *prox1*) did not yield hepatopancreas agenesis phenotypes (Fig. 3J; not shown). However under the same conditions, 38% (5/13) of the *Tg(hsp70l:dkk1-GFP)*^{w32} embryos in the *hnf1ba*^{s430} mutant background showed a loss of the entire hepatopancreas system, including the liver, ventral pancreas, and associated ducts (Fig. 3K). These hepatopancreas phenotypes were indistinguishable from those of *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants, though they occurred with greater penetrance. Importantly, we emphasize that all organ agenesis defects observed are permanent and do not recover at later stages, strongly supporting a specification defect rather than a general developmental delay. The complete loss of the entire hepatopancreas organ system in *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants and *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} embryos suggests a possible defect in specification of hepatopancreas progenitors. Consistently, heat-shocked *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} embryos lack early Prox1 and *hhex* expression in the ventral foregut at 36 hpf (a stage when their expression in both liver and pancreas progenitors are consistently detectable in wild type and single mutants) without detectable reduction of Pdx1 expression in the presumptive duodenum, indicating a specific loss of hepatopancreas progenitors (Fig. 4A-H; *n*=30). Interestingly, *prox1* and *hhex*, which we demonstrated to be downstream of Hnf1ba and Wnt signaling during hepatopancreas specification, have also been associated with increased diabetes risk by GWA studies (Dupuis et al., 2010). A genetic interaction among these factors in hepatopancreas development may hint at their potential interactions in contributing to diabetes. We note that heat-shock induction at later stages (23 hpf or later) dramatically decreased the penetrance of the hepatopancreas system agenesis phenotype, suggesting that initial

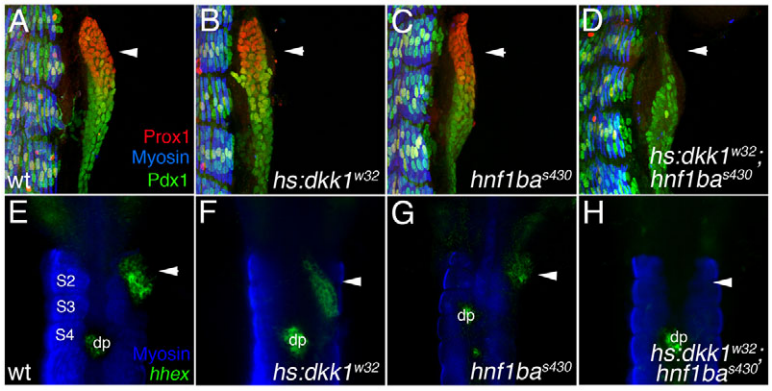

Fig. 4. Specification of hepatopancreas progenitors by Hnf1ba and Wnt signaling. (**A-D**) 3D rendering (lateral view showing somites for positional reference) of 36 hpf foregut endoderm in wild-type (A), *Tg(hsp70l:dkk1-GFP)*^{w32} (B), *hnf1ba*^{s430} (C) and *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} embryos (D) stained for Prox1, Pdx1 and myosin (somites). Relative to wild type, Prox1 foregut expression (arrowheads) is similar or mildly reduced in both *Tg(hsp70l:dkk1-GFP)*^{w32} and *hnf1ba*^{s430} embryos. Prox1 expression is severely reduced or lost in *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} embryos. (**E-G**) Double *in situ* hybridization and antibody staining (ventral view) for the early hepatopancreas progenitor marker *hhex* and myosin at 36 hpf. *hhex* is expressed (arrowheads) in the foregut endoderm and dorsal pancreas (dp) in wild type (E) but reduced in both the *Tg(hsp70l:dkk1-GFP)*^{w32} (F) and the *hnf1ba*^{s430} (G) embryos. (**H**) *hhex* expression (arrowhead) is undetectable in the foregut endoderm of *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*^{s430} embryos.

Wnt signaling between 21 and 23 hpf is crucial for hepatopancreas specification. And based on when Wnt inhibition was needed to efficiently block hepatopancreas specification, we suggest that between 21 and 28 hpf, just prior to liver budding, Wnt signaling is required for hepatopancreas progenitor specification. Blocking Wnt signaling at later stages (48 hpf) via ectopic *dkk1* expression led to a reduction in liver size (Goessling et al., 2008), suggesting a later role for Wnt signaling in liver expansion, distinct from its earlier role in hepatopancreas progenitor specification.

hnf1ba is necessary for Wnt signaling activity in the foregut endoderm

The highly specific loss of the hepatopancreas system in *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants suggests that Hnf1ba and Wnt2bb functions converge at the foregut endoderm to induce hepatopancreas fate. To determine the mechanism of this genetic interaction, we analyzed the expression of *hnf1ba*, *wnt2bb* and *prox1* at the earliest stage of hepatopancreas development (Sun and Hopkins, 2001; Ober et al., 2006; Huang et al., 2008; Lokmane et al., 2008; Noël et al., 2008). At 24 hpf, *hnf1ba* expression extends from the presumptive pancreas/liver to the presumptive duodenal regions of the foregut endoderm (from AP region of somite 1-5; Fig. 5A-C). At this stage, *wnt2bb* shows confined expression (somite 1-2) in the lateral plate mesoderm adjacent to the anterior-most region of the *hnf1ba* expression domain. Coincidently, it is in this anterior subdomain of *hnf1ba* expression that initial *prox1* expression becomes detectable. Together with our functional data, we hypothesize that *hnf1ba* expression defines the area of the foregut endoderm that can be induced by Wnt signaling to express Prox1 and adopt hepatopancreas fate (Fig. 5D). According to this model, broad overexpression of Wnt ligands should lead to posterior expansion of Prox1 expression where *hnf1ba* is expressed. As predicted and consistent with previous studies (Poulain and Ober, 2011; Shin et al., 2011), broad overexpression of Wnt8a via heat-shock of *Tg(hsp70l:wnt8a)*^{w34} embryos (Weidinger et al., 2005) beginning at 21 hpf resulted in a robust posterior expansion of Prox1 expression within the *hnf1ba*-positive domain of the foregut endoderm (Fig. 5E,F; *n*=30). Crucially, in *hnf1ba*$^{s430/hi2169}$ transheterozygotes, Prox1 expression is either not induced or is weakly induced in the foregut endoderm following ectopic Wnt8 expression (Fig. 5G; 100%, *n*=6; and inconsistently induced in *hnf1b*^{s430} homozygotes; not shown). These data demonstrate that *hnf1ba* function is indeed required for Wnt signaling to induce hepatopancreas fate. A possible mechanism for this genetic interaction is that *hnf1ba* is necessary for transduction of the Wnt signal in the foregut endoderm. To investigate this, we examined the expression of *cmyc* (*myca*), a direct Wnt/β-catenin target (He et al., 1998), which is expressed in hepatopancreas progenitors and whose expression domain can be induced posteriorly by ectopic Wnt8a expression (Fig. 5H) (Yamaguchi et al., 2005; Shin et al., 2011). Intriguingly, this posterior expansion also correlates with the *hnf1ba* expression domain (compare Fig. 5I with Fig. 5A). To test whether *cmyc* expression in the foregut is Wnt signaling dependent, we overexpressed the Wnt antagonist Dkk1, starting at 21 hpf and found a severe reduction in *cmyc* expression (*n*=25) (Fig. 5J). These data suggest that *cmyc* expression is a faithful marker of endogenous

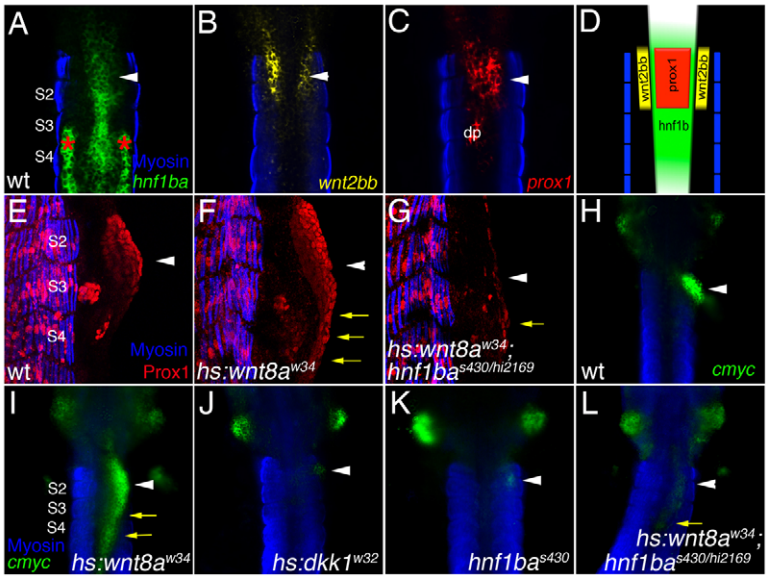

Fig. 5. Hnf1ba is required for Wnt signaling activity in the foregut endoderm. (**A-C**) Double antibody (myosin; blue) and *in situ* hybridization staining (ventral view) of 24 hpf wild-type foregut endoderm for expression of *hnf1ba* (A), *wnt2bb* (B) and *prox1* (C, arrowheads). (**D**) Illustration summarizing foregut expression domains showing restricted *prox1* expression within the anterior region of the *hnf1ba* foregut expression domain and adjacent to the *wnt2bb* expression domain in the lateral plate mesoderm. Pronephric *hnf1ba* expression (red asterisks in A) and dorsal pancreas *prox1* expression (dp) are not depicted in the illustration. (**E-G**) Three-dimensional rendering (lateral view) of 36 hpf foregut endoderm in wild-type (E), *Tg(hsp70l:wnt8a-GFP)*^{w34} (F) and *Tg(hsp70l:wnt8a-GFP)*^{w34}; *hnf1ba*$^{s430/hi2169}$ (G) embryos heat-shocked from 21-28 hpf at 37°C and stained for Prox1 and myosin. Compared with wild type, heat-shocked *Tg(hsp70l:wnt8a-GFP)*^{w34} embryos show significant posterior expansion of Prox1 in the foregut endoderm (yellow arrows; *n*=8/8). In *Tg(hsp70l:wnt8a-GFP)*^{w34}; *hnf1ba*$^{s430/hi2169}$ embryos, only weak or no Prox1 expression is observed (yellow arrows; *n*=6/6). (**H-L**) Double antibody and *in situ* hybridization staining (ventral view) of *cmyc* at 36 hpf in wild-type (H), *Tg(hsp70l:wnt8a-GFP)*^{w34} (I), *Tg(hsp70l:dkk1-GFP)*^{w32} (J), *hnf1ba*$^{s430-/-}$ (K) and *Tg(hsp70l:dkk1-GFP)*^{w32}; *hnf1ba*$^{s430/hi216}$ (L) embryos heat-shocked from 21 to 28 hpf. *cmyc* expression is restricted in the wild-type foregut endoderm (white arrowhead in H) but is expanded posteriorly in *Tg(hsp70l:wnt8a-GFP)*^{w34} (yellow arrows in I; *n*=12/12) or severely reduced in *Tg(hsp70l:dkk1-GFP)*^{w32} (white arrowhead in J) embryos. *cmyc* expression is reduced or lost in the *hnf1ba*^{s430} foregut endoderm (white arrowhead in K; *n*=4/4), and only weakly induced in *Tg(hsp70l:wnt8a-GFP)*^{w34}; *hnf1ba*$^{s430/hi2169}$ embryos (yellow arrow in L; *n*=4/4).

Wnt signaling activity in the foregut endoderm. In *hnf1ba*^{s430} homozygotes, *cmyc* foregut expression is markedly reduced, suggesting that *hnf1ba* function is important for Wnt signaling in the foregut endoderm (Fig. 5K; n>30). Furthermore, in *hnf1ba*$^{s430/hi2169}$ transheterozygotes, *cmyc* foregut expression cannot be robustly induced by ectopic Wnt8, suggesting that *hnf1ba* is important for foregut endoderm Wnt signaling activity (Fig. 5L; 100%, n=12; and inconsistently induced in *hnf1ba*^{s430} homozygotes). Because these results demonstrate that *hnf1ba* is required for the expression of a known transcriptional target of Wnt signal transduction, *cmyc*, we suggest that *hnf1ba* functions to generate a permissive domain in the foregut endoderm for Wnt signaling. A role for Hnf1ba in Wnt signaling could explain the severe, yet localized, foregut defect observed when combining the *wnt2bb*^{s404} and *hnf1ba*^{s430} mutations. In *wnt2bb*^{s404}; *hnf1ba*^{s430} double homozygous mutants, Wnt2bb loss of function would be compounded by the reduced foregut Wnt signaling that results from Hnf1ba partial loss of function. We suggest that this synergistic reduction of Wnt signaling contributes to the loss of hepatopancreas specification in *wnt2bb*^{s404}; *hnf1ba*^{s430} double mutants. It is possible that non-Wnt-related targets of Hnf1ba also contribute to the specification of hepatopancreas progenitors.

DISCUSSION

Our studies suggest that Hnf1ba and Wnt2bb coordinate the specification of the progenitor population that gives rise to the liver, ventral pancreas, gall bladder and associated ducts, but not neighboring endodermal tissue such as the dorsal pancreas, demonstrating a common genetic mechanism for specification of the entire ventrally derived hepatopancreas system. Although it has been suggested that genetic events regulating liver and pancreas specification occur during early somitogenesis (Chung et al., 2008; Wandzioch and Zaret, 2009), we show the Hnf1ba and Wnt signaling interact later, only several hours prior to liver budding, to induce hepatopancreas fate. It is well established that signals from the mesoderm play a key role in patterning the foregut endoderm during development (Kumar et al., 2003; Ober et al., 2006; Sneddon et al., 2012). But it is less clear how the endoderm regulates the reception of these signals. We provide *in vivo* evidence that Hnf1ba function is required for the foregut endoderm to respond to Wnt2bb signaling from the mesoderm.

We anticipate that this conceptual advance in understanding how hepatopancreas progenitors are normally specified could contribute to more efficient production of differentiated liver or pancreas cells to potentially treat liver disease and diabetes. HNF1B+ cells are produced in protocols designed to generate pancreatic endocrine cells from embryonic stem cells (Kroon et al., 2008). Based on our discoveries, we suggest that stimulating Wnt signaling in such HNF1B+ endoderm cells would increase the efficiency of current stem cell differentiation protocols by increasing the hepatopancreas progenitor pool. Furthermore, an *in vitro* study using human embryonic stem cells suggests that a restricted level of Wnt signaling, at a stage when HNF1B expression is robust, can enhance development of pancreas fate by 15-fold (Nostro et al., 2011), supporting a positive role for Wnt signaling in human pancreas development. This apparent functional conservation further validates zebrafish as a reliable vertebrate model for human pancreas development. Notably, our work suggests that there is a narrow developmental window when positive Wnt signaling input is required for specification of both liver and pancreas progenitors. Because Wnt signaling outside this window may have negative effects on pancreas specification (Murtaugh, 2008), precise stimulation of HNF1B+ endoderm cells by Wnt signaling in differentiation protocols may be necessary. Future studies to determine how progenitors specified by Hnf1b and Wnt signaling are subsequently allocated into distinct liver, pancreas, gall bladder, or duct fate will be important to further enhance stem cell differentiation efforts.

Despite the discovery of MODY5 over 14 years ago, the role HNF1B plays in pancreas development and diabetes has remained elusive, hindered by the lack of a suitable animal model system. Homozygous *Hnf1b*-null mutants exhibit broad defects that preclude pancreas and liver morphogenesis studies. Furthermore, heterozygous null *Hnf1b* mutants were not reported to exhibit MODY5-like pancreas defects, suggesting that *Hnf1b* dose regulation and/or requirements are different among vertebrates. Our studies demonstrate the utility of a homozygous hypomorphic *hnf1ba* mutant model that mimics pancreas pathologies observed in humans with heterozygous *HNF1B* mutations. Interestingly, variation in the severity of pancreas hypoplasia found in this *hnf1ba* zebrafish model is also characteristic of MODY5 cases (Bellanné-Chantelot et al., 2004). This variation is even found among patients within the same family (Barbacci et al., 2004; Edghill et al., 2006a). We postulate that, like homozygous *hnf1ba*^{s430} zebrafish mutants, heterozygous *HNF1B* mutations in individuals with MODY5 result in a reduction of HNF1B function to a crucial threshold that is sensitive to stochastic developmental variation (Gärtner, 1990; Baranzini et al., 2010; Raj et al., 2010), which can be sensitive to variations in genetic background. Using this novel MODY5 model, we reveal that reduced Hnf1ba function can compromise developmental β-cell numbers, and show that this reduction is not secondary to pancreas hypoplasia defects. Furthermore, as we find in *hnf1ba*^{s430} zebrafish mutants, exocrine pancreas hypoplasia and islet disorganization were also observed in a study of two human fetuses harboring heterozygous *HNF1B* mutations (Haumaitre et al., 2006). These common defects suggest that Hnf1b function in pancreas development is broadly conserved among vertebrates. However, a reduction in the number of β-cells was not reported in that study. We suggest that, similar to the variation in pancreas hypoplasia in both individuals with MODY5 and zebrafish *hnf1ba*^{s430} homozygotes, a decrease in embryonic β-cell numbers in MODY5 is also variable – which may explain the variable onset of MODY5 diabetes. In support of this idea, rare cases of neonatal diabetes have been reported for MODY5, suggesting that β-cell numbers may be developmentally compromised in some people with *HNF1B* mutations (Edghill et al., 2006b; Beckers et al., 2007). In addition to the previously suggested roles for Hnf1b in β-cell insulin secretion and hepatic insulin signaling (Wang et al., 2004; Brackenridge et al., 2006; Kornfeld et al., 2013), our findings raise the possibility that a reduction in β-cell number may be a significant contributing factor to the etiology of HNF1B diabetes, potentially contributing to insulin dependence in MODY5. Furthermore, using this hypomorphic Hnf1b mutant model, we provided definitive evidence that *hnf1ba* plays a key role in ventral pancreas specification (Haumaitre et al., 2005), upstream of *ptf1a* and distinct from its roles in regulating foregut regionalization and liver specification. By revealing specific roles for *hnf1ba* in β-cell and pancreas development, our findings set the stage for future work to explore the mechanism of these crucial functions.

Our studies revealing an unexpected coordinate role of *hnf1ba* with *wnt2bb* in hepatopancreas specification also lays the foundation for future research to explore how Hnf1ba, as a transcription factor, functions to generate a Wnt permissive foregut endoderm and whether this genetic interaction is conserved in other

tissues or in disease. Hnf1ba could be functioning in parallel with Wnt signaling by independently regulating Wnt target genes or upstream of Wnt signaling by regulating a component of the Wnt signal transduction pathway, including co-factors. Previous biochemical studies have suggested that Hnf1b can regulate target genes via epigenetic modification (Barbacci et al., 2004; Verdeguer et al., 2010). Future studies that seek to determine the precise molecular nature of this genetic mechanism will need to investigate these avenues of Hnf1b function. An understanding of this interaction may also have implications for their potential interaction in disease, as GWA studies have revealed that both *HNF1B* and the Wnt effector gene *TCF7L2* are strongly associated with T2D (Frayling, 2007). With our finding that Hnf1ba is required for expression of the direct Wnt signaling target *cmyc*, we suggest that a possible consequence of HNF1B deficiency in diabetes may be the attenuation of Wnt signaling. Consistent with this model, a human hypoplastic MODY5 fetal pancreas was shown to have decreased expression of a Wnt signaling component β-catenin (Haumaitre et al., 2006).

The hypomorphic *hnf1ba*^{s430} allele presented here will be a valuable model for further investigations into the genetic mechanisms of embryonic and post-embryonic pathologies associated with MODY5, including maturity onset diabetes and pancreas, liver and kidney dysfunctions. Because mouse *Hnf1b*-null heterozygotes were not reported to have MODY5-like phenotypes, we suggest that a mammalian model with a homozygous *Hnf1b* hypomorphic mutation similar to *hnf1ba*^{s430} may be necessary to phenocopy MODY5 pancreas defects. This approach of using hypomorphic alelles was shown to be necessary to model heterozygous *SOX2* mutations in humans with anophthalmia-esophageal-genital (AEG) syndrome (Que et al., 2007). Importantly, an *Hnf1b* mouse model with a broad and partial reduction in Hnf1b function would be more comparable with the MODY5 genetic condition than would a conditional *Hnf1b* knockout model. Such a mouse would be valuable to complement our findings on the functions of Hnf1b in vertebrate hepatopancreas progenitor specification, particularly if combined with precise Wnt signaling inhibition.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
P.D.S.D. made original observations, generated initial data and hypotheses, and submitted a manuscript while in D.Y.R.S.'s lab; P.D.S.D. and J.J.L. designed and executed experiments, and assembled figures; K.P.G. and D.Z. contributed to expression studies; P.D.S.D., N.Z. and T.K. contributed to mapping and cloning of the *liger*^{s430} mutation in D.Y.R.S.'s lab; J.J.L. and L.S. genotyped mutants; H.V. and P.D.S.D. contributed to mutagenesis screen in D.Y.R.S.'s lab; K.S. and C.V.E.W. produced and characterized the zebrafish Pdx1 antibodies; Y.C. provided protein structure analysis of the *liger*^{s430} mutation; P.D.S.D. and D.Y.R.S. oversaw studies; U.S.J and R.K.H performed transcriptional analysis, P.D.S.D., J.J.L. and D.Y.R.S. wrote the manuscript with feedback from C.V.E.W., K.P.G., D.Z., U.S.J., N.Z., K.S., R.K.H., T.K., L.S., H.V. and Y.C.

Supplementary material
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