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Crucial role of vHNF1 in vertebrate hepatic specification

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Mouse liver induction occurs via the acquisition of ventral endoderm competence to respond to inductive signals from adjacent mesoderm, followed by hepatic specification. Little is known about the regulatory circuit involved in these processes. Through the analysis of *vHnf1* (*Hnf1b*)-deficient embryos, generated by tetraploid embryo complementation, we demonstrate that lack of vHNF1 leads to defective hepatic bud formation and abnormal gut regionalization. Thickening of the ventral hepatic endoderm and expression of known hepatic genes do not occur. At earlier stages, hepatic specification of *vHnf1*$^{-/-}$ ventral endoderm is disrupted. More importantly, mutant ventral endoderm cultured in vitro loses its responsiveness to inductive FGF signals and fails to induce the hepatic-specification genes albumin and transthyretin. Analysis of liver induction in zebrafish indicates a conserved role of vHNF1 in vertebrates. Our results reveal the crucial role of vHNF1 at the earliest steps of liver induction: the acquisition of endoderm competence and the hepatic specification.

KEY WORDS: Liver specification, vHNF1 (HNF1β, TCF2), Mouse, Zebrafish

INTRODUCTION

During embryogenesis, the primitive gut forms during gastrulation and is gradually patterned along its anterior-posterior axis into the fore-, mid- and hindgut domains. The foregut contains the common endodermal precursors of the liver, pancreas, thyroid and lung. Reciprocal tissue interactions between the endoderm and adjacent mesoderm further subdivide these broad primitive gut domains into smaller subdomains, and allow for the emergence of different endoderm-derived organs at precise locations. The mammalian liver is derived from both the endoderm of the ventral foregut and the adjacent mesenchyme of the septum transversum. In the mouse, its development begins at 8 days post-conception (E8), when the ventral foregut lies immediately adjacent to the cardiac mesoderm. Liver development is thought to occur in sequential steps, beginning with the acquisition of competence to respond to inductive signals, followed by hepatic specification and bud formation. FGFs from the cardiac mesoderm and BMPs from the septum transversum mesenchyme (STM) coordinately induce the underlying endoderm to adopt a hepatic fate (Jung et al., 1999; Rossi et al., 2001). The hepatic endoderm responds to this induction by expressing albumin (*Alb*), transthyretin (*Ttr*) and alpha fetoprotein (*Afp*), and generates a nascent hepatoblast population (bipotential precursors) from which the hepatocytes and biliary cells are derived (Lemaigre and Zaret, 2004). By E9, hepatoblasts proliferate to form the primary liver bud. The basement membrane surrounding the liver bud is then degraded, and hepatoblasts delaminate from the bud and invade the surrounding STM in a cord-like fashion (McLin and Zorn, 2006). Aside from the inductive signals involved in early liver development, little is known about the transcriptional regulatory circuit within the ventral endoderm itself that controls hepatic specification.

Gene-targeting studies have implicated several transcription factor genes in controlling distinct aspects of early hepatogenesis in the endoderm, including *Hex* (*Hhex* – Mouse Genome Informatics), *Gata6* and *Prox1*. In *Hex*-deficient embryos, the hepatic diverticulum is specified but proliferation is reduced and subsequent migration of hepatoblasts into the STM fails to occur (Bort et al., 2004; Martinez Barbera et al., 2000). Similarly, in *Gata6*$^{-/-}$ embryos, hepatic development is disrupted at the liver bud stage soon after hepatic specification (Zhao et al., 2005). The homeodomain transcription factor PROX1 acts at later stages. Hepatoblasts differentiate but are unable to delaminate into the adjacent STM; this is most likely owing to their inability to degrade the basement membrane surrounding the hepatic bud (Sosa-Pineda et al., 2000). Thus, although mutations in these transcription factors affect early liver development, in each case defects occur shortly after hepatic induction. Defective hepatic specification has only been observed in a double-mutant mouse model in which both *Foxa1* and *Foxa2* are deleted at the onset of liver induction (Lee et al., 2005). Interestingly, ventral endoderm explants of these compound mutants are unable to induce hepatogenesis in vitro, thus providing genetic support for the requirement of Foxa factors for hepatic competence. By contrast, constitutive inactivation of *Foxa1* or ablation of *Foxa2* specifically in the ventral endoderm does not affect normal hepatic specification or differentiation.

Recent studies in mouse and zebrafish indicate that the homeodomain transcription factor vHNF1 (HNF1β, TCF2) plays a crucial role in the early development of several endodermally derived organs (Haumaitre et al., 2005; Sun and Hopkins, 2001). *vHnf1*-deficient mouse embryos die before gastrulation owing to defective formation of extraembryonic visceral endoderm. This early embryonic lethality can be rescued by providing *vHnf1*-null embryos with a wild-type (WT) extraembryonic endoderm through the use of tetraploid embryo complementation (Barbacci et al., 1999; Haumaitre et al., 2005). Using this technique, we have previously shown that vHNF1 is required for ventral pancreas induction, as well as for normal dorsal pancreas morphogenesis and regional specification of the gut (Haumaitre et al., 2005). Rescued *vHnf1*-deficient embryos also exhibit a severe liver hypoplasia, the molecular basis of which

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remains essentially unexplored. Interestingly, zebrafish *vhnf1* hypomorphic mutants also exhibit abnormal gut, liver and pancreas development (Sun and Hopkins, 2001), suggesting that the function of vHNF1 is conserved through vertebrate evolution. In this study, we have examined the role of *vHnf1* in early hepatic development in both mouse and zebrafish *vHnf1* mutant embryos. Our results uncover the requirement of vHNF1 for liver specification in both organisms. More importantly, using an explant culture system, we have also established that *vHnf1*-deficient mouse ventral endoderm is unable to respond to FGF signals, which have previously been shown to be sufficient to induce hepatic specification in vitro (Calmont et al., 2006; Serls et al., 2005). Therefore, *vHnf1* is required for the initiation of liver development in vertebrates.

MATERIALS AND METHODS

Generation of ES cell-derived mouse embryos

Tetraploid (4n) embryos were generated as previously described (Haumaitre et al., 2005). The genotype of 4n embryos was confirmed by PCR analysis of genomic DNA and no WT allele was detected in embryos derived from *vHnf1*$^{-/-}$ ES cells. Control embryos were obtained by parallel matings (OF1 females × B6D2F1 males). We used embryos with identical numbers of somites to ensure that they were at equivalent developmental stages.

Immunohistochemistry and in situ hybridization

Antibody staining was performed as previously described (Haumaitre et al., 2005). We used the following primary antibodies: mouse anti-E-cadherin at 1:100 (BD Transduction Laboratories), rabbit anti-laminin at 1:50 (Sigma), goat anti-HNF4α at 1:150 (C-19, Santa Cruz), rabbit anti-vHNF1 at 1:50 (H-85, Santa Cruz), rabbit anti-NKX2.1 at 1:100 (H-190, Santa Cruz) and rabbit anti-phosphohistone H3 at 1:250 (Upstate Biotechnology). Secondary antibodies were goat anti-mouse IgG Alexa Fluor 546 at 1:500, donkey anti-rabbit IgG Alexa Fluor 488 at 1:500 (Molecular Probes) and biotinylated goat anti-rabbit IgG, horse anti-mouse IgG and anti-goat IgG (Vector) followed by streptavidin-Alexa Fluor 488 at 1:500 (Molecular Probes). TUNEL staining was performed using the Cell Death Detection Kit (Roche). Whole-mount in situ hybridization or on sections was performed as previously described (Haumaitre et al., 2005), with the exception that embryos were fixed with 60% ethanol/11% formaldehyde and 10% acetic acid. The following cRNA probes were used: *vHnf1* (Haumaitre et al., 2005), *Afp*, *Prox1* (G. Gradwohl, Inserm U682, Strasbourg, France), *Foxa2*, *Foxa1* and *Foxa3* (K. H. Kaestner, Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA), *Shh* (A. P. McMahon, Harvard University, Cambridge, MA), *Hex* (Bedford et al., 1993), *Gata6* and *Gata4* (D. B. Wilson, Washington University Medical Center, St. Louis, MO); *Irx2*, *Hnf4a*, *Gata1* and *Hlx* probes were generated by PCR.

RNA extraction, RT-PCR, embryo tissue isolation and culture

RNA from dissected ventral endoderm or after its in vitro culture was extracted using the RNeasy Micro Kit (Qiagen) and subjected to semi-quantitative RT-PCR as described (Barbacci et al., 1999). The volume of each cDNA preparation was adjusted to give similar exponential phase PCR signal for *Gapdh*. Primer sequences are available upon request. For explant culture experiments, ventral endoderm was dissected from 4- to 6-somite embryos and cultured for 48 hours at 37°C in 4 microwells (Nunc) coated with type I collagen (Collaborative Biomedical Products) in 5% CO_2 in DMEM medium supplemented with 10% calf serum, human recombinant bFGF (Invitrogen; 5 ng/ml) and heparan sulfate proteoglycan (Sigma; 50 ng/ml).

Zebrafish line maintenance, genotyping and whole-mount in situ hybridization

Zebrafish (*Danio rerio*) were raised and genotyped as described (Lecaudey et al., 2007). Whole-mount in situ hybridization was performed using the following probes: *hhex* and *transferrin* (M. Pack, The Children's Hospital of Philadelphia, Pennsylvania, PA), *gata 4 and gata6* (T. Evans, Albert Einstein College of Medicine, New York, NY), *foxa3, ceruloplasmin* and *prox1* (Y. R. Stainier, University of California, San Fransisco, CA) *axial* (*foxa2*) and *shh* (F. M. Rosa, Inserm U784, Paris, France).

RESULTS

vHnf1 is expressed in the developing mouse liver from its earliest stages

As a first step towards addressing the role of vHNF1 in liver development, we carried out a detailed analysis of its expression in mouse embryos from the first stages of its development. In situ hybridization on 7- to 8-somite pair stage (7-8s) embryos revealed low levels of *vHnf1* transcripts in the ventral endoderm adjacent to the developing heart, in a region that delineates the future hepatic and ventral pancreas endoderm (Fig. 1A). At the hepatic bud stage (E9.5; 20s), *vHnf1* was strongly expressed in the hepatic primordium (Fig. 1B). At the stage during which the hepatoblasts begin to invade the adjacent STM, *vHnf1* transcript levels decreased in these cells, whereas in the gall bladder primordium the levels remained very high (Fig. 1C). At this stage, *vHnf1* transcripts were also detected in the gut, the lung buds, the ventral and dorsal pancreas and the posterior stomach (Fig. 1D). By E17.5-18.5, *vHnf1* expression increased transiently in parenchymal hepatocytes and sharply decreased after birth [around post-natal day 10 (P10)]; in the adult, *vHnf1* expression was restricted to cholangiocytes (data not shown) (Kyrmizi et al., 2006). Thus, *vHnf1* expression demarcates the early hepatic and ventral pancreas endoderm, which is consistent with a function of this factor during the earliest stages of development.

Lack of vHNF1 leads to a severe defect in mouse liver development

We have rescued the early embryonic lethality of *vHnf1* homozygous mutants by providing WT extraembryonic endoderm through tetraploid embryo complementation. In these experiments, the embryo proper is derived exclusively from *vHnf1*$^{-/-}$ diploid ES cells and the visceral endoderm derives from WT tetraploid embryos (Barbacci et al., 1999). *vHnf1*$^{-/-}$ ES cell-derived embryos (henceforth denoted

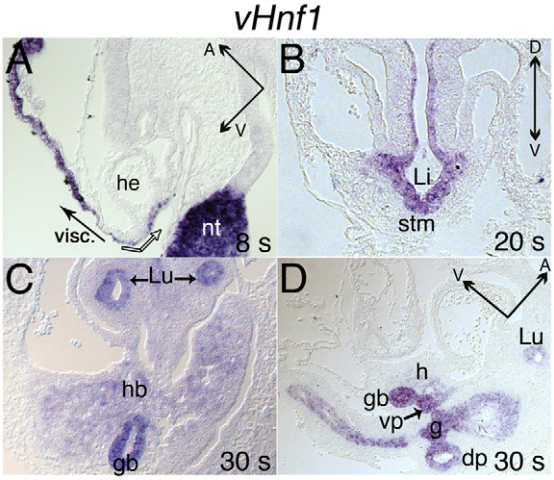

Fig. 1. *vHnf1* expression in the developing mouse liver. (**A-D**) In situ hybridization identifying *vHnf1* expression in developing liver of sagittal (A,D) or transversal (B,C) sections of wild-type (WT) mouse embryos at 8s (A), 20s (B) and 30s (C,D). (A) *vHnf1* transcripts are visualized in the ventral hepatic endoderm (white arrow), in the visceral endoderm (visc., black arrow) and at higher levels in the neural tube (nt). (B) *vHnf1* is strongly expressed in the liver bud (Li) invading the adjacent septum transversum mesenchyme (stm). (C) *vHnf1* is strongly expressed in the lung bud (Lu) and in the gall bladder (gb), but is expressed at much lower levels in the migrating hepatoblast (hb). (D) Strong *vHnf1* expression is observed in the entire gut (g), ventral and dorsal pancreas (vp, dp), gall bladder (gb), lung buds (Lu) and hepatoblasts (hb). Axes: A, anterior; D, dorsal; V, ventral.

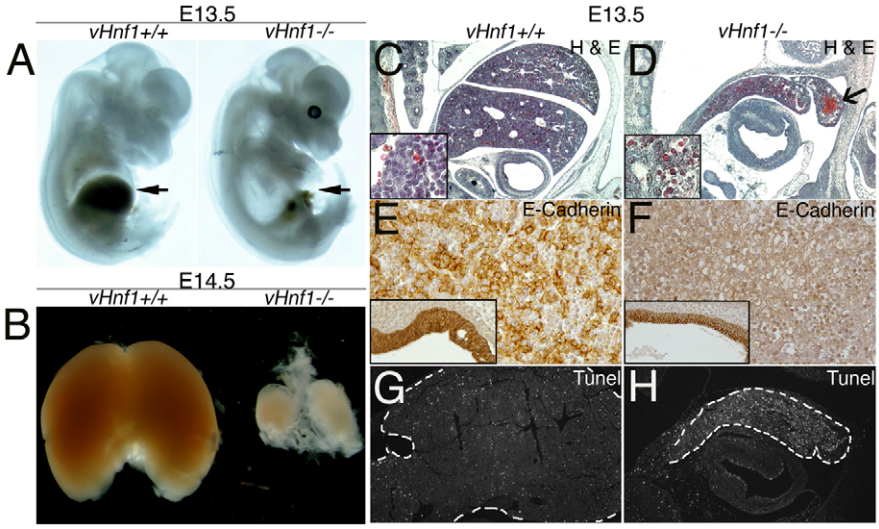

Fig. 2. Severe liver hypoplasia in *vHnf1*$^{-/-}$ embryos. (**A**) Direct observation of WT and *vHnf1*$^{-/-}$ mouse embryos at E13.5 shows an essentially empty abdominal cavity in the mutants (arrows). (**B**) Liver dissection at E14.5 reveals the liver hypoplasia of mutant embryos. (**C**,**D**) Hematoxylin and Eosin (H&E) staining of sagittal sections of WT (C) and *vHnf1*$^{-/-}$ (D) embryos at E13.5 show the reduced volume of mutant liver and the presence of hemorrhagic regions (arrow). At higher magnification, the strong disorganization of mutant liver tissue is evident (compare insets). (**E**,**F**) Immunostaining at E13.5 indicates the absence of E-cadherin expression in mutant liver (F), in contrast to WT (E) in which the staining reveals the epithelial organization of hepatoblasts. The insets illustrate correct E-cadherin expression in the stomach epithelium of WT (E) and of mutant (F) embryos. (**G**,**H**) TUNEL analysis at E13.5 shows a massive apoptosis of the majority of cells that compose the mutant liver (H), whereas in WT embryos few apoptotic cells are observed (G). The dashed line outlines the liver.

vHnf1$^{-/-}$) exhibited apparently normal external characteristics up to E13.5, except for severe liver hypoplasia, evident by external inspection (Fig. 2A). Dissection of mutant livers indicated a 70% reduction in size; yet they presented the same number of lobes as WT livers (Fig. 2B). *vHnf1*$^{-/-}$ embryos died around E14.5-15.5, probably because of the inability of the hypoplastic mutant liver to conduct its embryonic hematopoietic function.

Histological analysis at E13.5 confirmed that the *vHnf1*$^{-/-}$ liver occupied a very reduced volume in the abdominal cavity. The liver architecture was severely disorganized with ventrally located hemorrhagic regions (Fig. 2D, arrow). Moreover, cells were rounded and lacked hepatoblast characteristics (Fig. 2D, insets). The absence of the cell-adhesion protein E-cadherin (cadherin 1) in *vHnf1*$^{-/-}$ livers further suggested a defect in the epithelial characteristics of the hepatoblasts (Fig. 2F), or their absence. The gall bladder primordium, which normally develops in conjunction with the liver, was not discernible at any stage (data not shown).

Analysis of the percentage of cells positive for the mitotic marker phosphohistone H3 (P-H3) showed a 60% decrease in P-H3-positive cells in *vHnf1*$^{-/-}$ relative to WT livers between E10.5 and E12.5, and a decrease of 60-70% by E13.5 (not shown). Whereas no increase in apoptosis was detected in mutant livers from E10.5 to E12.5, massive apoptosis was detected at E13.5 (Fig. 2H).

Considering that *vHnf1* is selectively expressed in the gut endoderm and hepatoblasts, but not in the STM, we examined the expression of early endoderm differentiation markers, including *Alb*, *Afp*, *Ttr*, *Prox1*, *Foxa1*, *Foxa2*, *Foxa3*, *Hnf1a*, *Hnf4a* and *Hnf6* (*Onecut1* – Mouse Genome Informatics), at earlier stages, before liver damage was evident. The combined expression of these genes would reflect correctly differentiated hepatoblasts. Surprisingly, none of these markers was expressed at E10.5, nor at any stage (Fig. 3A-L, and data not shown), in the *vHnf1* mutant liver.

We then examined the expression of markers of other cells types that compose the embryonic liver, namely the STM, endothelial and hematopoietic cells. In the embryonic liver, the homeobox transcription factor *Hlx* is expressed exclusively in cells derived from the STM (Hentsch et al., 1996). Once hepatoblasts invade the STM, *Hlx* expression essentially becomes restricted to the periphery of hepatic lobes (E10.5-11; Fig. 3M). Surprisingly, *Hlx* expression at E10.5 was observed homogenously throughout the entire lobes of *vHnf1*$^{-/-}$ livers (Fig. 3N), along with reduced expression of *Gata1* (Fig. 3P), a transcription factor restricted to hematopoietic cells (Palis et al., 1999). Together, these results show that mutant hepatic lobes are devoid of hepatoblasts and are mainly composed of mesenchymal and hematopoietic cells, which enter into apoptosis by E13.5. These observations further suggest a defect in hepatoblast formation and/or migration in *vHnf1*$^{-/-}$ embryos.

vHNF1 is necessary for hepatic bud formation

Whole-mount in situ hybridization at E9.5 (20-22s) showed that *Afp*, one of the first markers expressed at high levels in the hepatic bud (Fig. 4A), was absent from the *vHnf1*$^{-/-}$ mutant presumptive hepatic domain (Fig. 4B). *Foxa2*, a gene normally expressed in the foregut-midgut endoderm and necessary for its development (Dufort et al., 1998), was specifically absent from the presumptive hepatic endodermal region, while in the gut endoderm its expression was caudally expanded (Fig. 4C,D). In the *vHnf1*$^{-/-}$ mutants, the expression of *Shh*, a foregut marker, was expanded and higher in the foregut region compared with WT embryos (Fig. 4E,F), which was more obvious on sagittal sections (not shown).

In situ hybridization of transversal sections at E9.5 (20-22s) showed that the expression of *Hex*, one of the earliest factors expressed in prehepatic endoderm and required for hepatic and ventral pancreatic bud development (Bort et al., 2004; Martinez Barbera et al., 2000), was barely detectable in the ventral endoderm

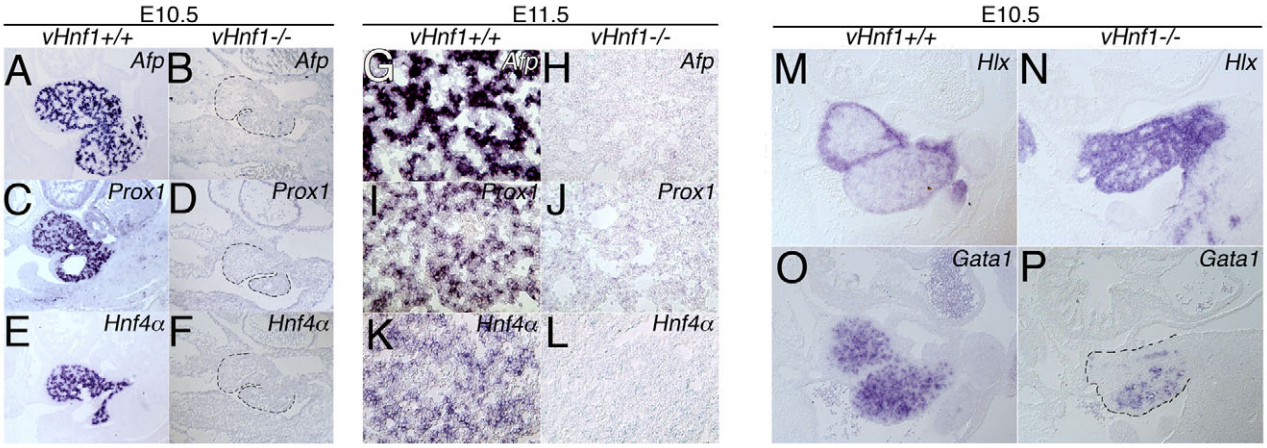

Fig. 3. Mesenchymal lobes devoid of hepatoblasts are formed in the absence of vHNF1. (**A-P**) In situ hybridization analysis of early liver differentiation markers at E10.5 (A-F) and E11.5 (G-L), mesenchymal (M,N) and hematopoeitic (O,P) markers at E10.5 in WT (A,C,E,G,I,K,M,O) and *vHnf1*$^{-/-}$ (B,D,F,H,J,L,N,P) mouse embryos. *Afp*, a specification marker, is strongly expressed in WT hepatoblasts at E10.5 (A) and E11.5 (G) but is totally absent from the mutant liver (B,H). *Prox1* (C,I) and *Hnf4a* (E,K) are also expressed in the WT but are not detected in the mutant at these stages (D,J,F,L). *Hlx*, a mesenchymal marker, is mainly expressed at the periphery of WT hepatic lobes at E10.5 (M). In the mutant, the expanded *Hlx* expression observed (N) indicates that the lobes are essentially formed by this cell type. Hematopoeitic cells, marked by *Gata1*, are present in greater numbers in WT (O) than mutant (P) hepatic lobes.

of *vHnf1*$^{-/-}$ embryos (Fig. 4G,H). Yet, *Hex* expression was not affected in the thyroid domain (data not shown). At this stage, migration of hepatoblasts is manifested by progressive disruption of the laminin-positive basal membrane that surrounds the liver bud of WT embryos (Fig. 4I). As in *Hex* mutants, in *vHnf1*$^{-/-}$ embryos the formation of a pseudostratified epithelial hepatic bud was not observed. The ventral endoderm remained columnar as the epithelium lining the gut tube and was surrounded by an intact laminin basal membrane (Fig. 4J). In agreement with the lack of endoderm outgrowth and expansion, we observed a 40% decrease in the number of P-H3^{+} cells in the presumptive *vHnf1*$^{-/-}$ hepatic endoderm (4.4±1.29%; 17 sections, *n*=2 embryos) as opposed to WT controls (7.49±1.35%; 16 sections, *n*=2 embryos) (data not shown). At E9.5, *Shh* is normally expressed in the lateral gut endoderm in a ventral-dorsal gradient but is excluded from the hepatic bud (Fig. 4K). In *Hex*$^{-/-}$ embryos, ectopic expression of *Shh* in the hepatic bud has been proposed to impair the transition of the endoderm to a pseudostratified epithelium (Bort et al., 2006). *Shh* was expressed ectopically and at higher levels in *vHnf1*$^{-/-}$ than WT embryos in the lateral gut epithelium (Fig. 4, compare E,K with F,L); it was however excluded from the presumptive hepatic bud domain.

Expression of *Prox1* was barely detectable in the ventral endoderm (Fig. 4N). By contrast, *Gata6* was expressed normally in the gut epithelium and adjacent STM of *vHnf1*$^{-/-}$embryos (Fig. 4Q,R). *Gata4* is normally expressed transiently in undifferentiated gut endoderm, which becomes competent to activate liver genes (Bossard and Zaret, 1998), but from E9 (14s) is rapidly downregulated in the prehepatic endoderm while persisting in the gut duodenal region (Bort et al., 2006) (see also Fig. S1 in the supplementary material). Surprisingly, *Gata4*, which was, as in the WT, induced at 8s and downregulated by 14s in mutant ventral endoderm (see Fig. S1 in the supplementary material), was subsequently ectopically expressed in the entire ventral gut of *vHnf1*$^{-/-}$ embryos (Fig. 4P and see S1 in the supplementary material). Interestingly, a few cells coexpressing GATA4 and the pancreatic-duodenal factor PDX1 were observed in a restricted part of the mutant ventral gut (see Fig. S1 in the supplementary material).

Remarkably, the expression of *Foxa1* and *Foxa2* was strongly decreased and/or absent in the ventral part of the gut and in the presumptive liver bud of *vHnf1*$^{-/-}$ embryos (Fig. 4T,V), whereas their expression in the lateral gut epithelium was unaffected. Furthermore, the expression of *Foxa3* was completely lost (Fig. 4X), which is in agreement with previous data indicating that vHNF1 is a direct transcriptional activator of this gene in visceral endoderm formation (Barbacci et al., 1999; Hiemisch et al., 1997).

These results indicate that vHNF1 is required in two steps of early liver development: the thickening of the hepatic bud and the expression of essentially all known hepatic genes.

vHNF1 is required for hepatic specification of the ventral endoderm

The observation that the expression of Foxa factors is strongly reduced or absent in the presumptive hepatic endoderm of *vHnf1*$^{-/-}$ embryos suggested a defective hepatic specification. To directly address whether the specification process was impaired, we determined the expression levels of several hepatic genes in ventral endoderm at the 6-8s stage by semi-quantitative RT-PCR (Fig. 5A). At this stage, the ventral foregut endoderm is instructed by FGF and BMP signals to be specified to the hepatic lineage. As expected, *vHnf1* transcripts were detected in ventral endoderm of control embryos, but not in *vHnf1*$^{-/-}$ ventral endoderm. Comparable *Hex* and *Foxa2* transcript levels were detected in mutant and WT embryos. By contrast, no transcripts of *Alb* were detected in mutant ventral endoderm (Fig. 5A). Since the *Alb* gene encodes the earliest marker expressed in the prehepatic endoderm, our results demonstrate that vHNF1 is required for hepatic specification. The same results were obtained when these analyses were performed at the 8-10s (Fig. 5B) or 10-12s stage (not shown), thereby excluding the possibility of a delay in hepatic specification. Furthermore, transcripts of *Ptf1a*, a key transcription factor required for the acquisition of a pancreatic fate (Kawaguchi et al., 2002), were not detected in mutant ventral endoderm at the 8-10s stage (Fig. 5B). These results show that ventral pancreas specification does not occur in *vHnf1*$^{-/-}$ mutants. This is in agreement with the reported absence

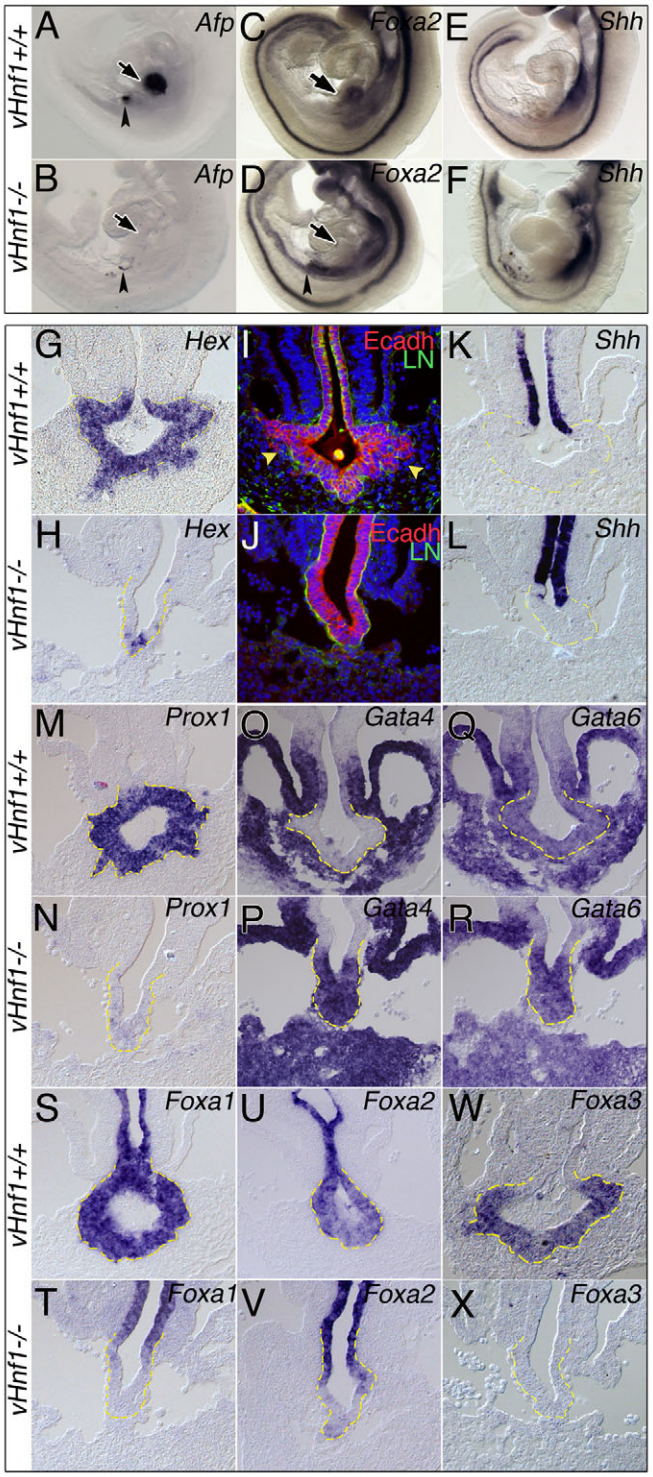

Fig. 4. vHNF1 is necessary for hepatic bud formation and expansion. (**A-F**) Whole-mount in situ hybridization analysis at 20-22s. *Afp*, strongly expressed in the WT mouse liver bud (A, arrow), is absent from the *vHnf1*$^{-/-}$ presumptive hepatic bud (arrow, B). Note that *Afp*, the expression of which in the visceral endoderm depends on *vHnf1* (Barbacci et al., 1999), was expressed at normal levels in the visceral endoderm of mutant embryos (arrowhead) because this tissue is derived from 4n WT embryos. *Foxa2* is expressed in the WT liver bud and the foregut endoderm (C), but its expression is lost in the presumptive mutant hepatic domain (arrow, D), along with a caudally expanded expression in the gut (arrowhead, D). *Shh* expression in the foregut endoderm is greatly enhanced in mutant embryos (F), but is excluded from the WT liver bud (E) and presumptive mutant hepatic domain (F). (**G-X**) In situ hybridization (G,H,K-X) and immunostaining (I,J) analyses on transversal sections at 20-22s. *Hex* is strongly expressed in the WT liver bud (G), whereas in the mutant only a few *Hex*-positive cells are detected in the presumptive liver bud (H). Immunostaining in a control embryo shows hepatic bud formation with E-cadherin (Ecadh) expression and hepatoblast delamination into the adjacent STM with disruption of laminin (LN)-positive basal membrane (arrowheads, I). In mutants, the endoderm does not form a pseudostratified epithelium, leading to the absence of a hepatic bud (J). *Shh* expression is excluded from the presumptive mutant liver bud (L) as in the WT liver bud (K) with a stronger and expanded expression in the lateral foregut. *Prox1*, which labels hepatoblasts (M), is absent from the *vHnf1*$^{-/-}$ ventral endoderm (N). *Gata4* is excluded from the ventral endoderm (O) but is ectopically expressed throughout the entire ventral gut of mutant embryos (P). No differences in *Gata6* expression are observed between WT and *vHnf1*$^{-/-}$ mutant (Q,R). Absence or strongly decreased expression of *Foxa1*, *Foxa2* and *Foxa3* is observed in the ventral part of the mutant gut (T,V,X) as compared with the control (S,U,W).

in *vHnf1*$^{-/-}$ mutants of a ventral pancreas bud at all stages examined (Haumaitre et al., 2005), and further indicates a global defect in ventral endoderm specification.

Impaired hepatic specification in *vHnf1* mutants could be due to a defective positioning of the definitive endoderm beyond the hepatogenic influence of the cardiac mesoderm, as described in *Hex*$^{-/-}$ mutants (Bort et al., 2004). We therefore compared the expression of vHNF1 and HNF4α proteins, which at 8s are both strongly expressed in the extraembryonic visceral endoderm. We found, however, a similar positioning of this tissue relative to the ventral foregut endoderm and cardiac mesoderm in *vHnf1*$^{-/-}$ and WT embryos (Fig. 5, compare C with D and E with F).

Another possible explanation for defective hepatic specification is a switch in the identity of the ventral endoderm to another, more-anterior fate of the foregut, as might be expected from the posterior expansion of *Foxa2* (Fig. 4D) and *Shh* (Haumaitre et al., 2005) expression in the gut of *vHnf1*$^{-/-}$ embryos. We analyzed at 8s the expression of several factors typically expressed in different ventral foregut regions, including *Nkx2.1*, which is expressed in the thyroid and lung domain (Deutsch et al., 2001), *Hex*, which is expressed in the thyroid and hepatic endoderm domain (Martinez Barbera et al., 2000), and *Irx2*, which is expressed in the lungs and trachea-pharyngeal domain (Becker et al., 2001). No differences in the expression pattern of these markers were observed between mutant and WT embryos, indicating that at this stage, *vHnf1*$^{-/-}$ ventral endoderm is apparently correctly regionalized (Fig. 5G-L). We also observed correct expression of *Hex* in the presumptive hepatic domain of mutant ventral endoderm. However, and in contrast to the similar levels of *Foxa1/2* transcripts observed by RT-PCR analysis of control and mutant ventral endoderm, the expression of both *Foxa1* and *Foxa2* appeared significantly reduced specifically in the mutant ventral endoderm region (Fig. 5N,P). One possible explanation for this discrepancy is that during ventral endoderm microdissection, some foregut-adjacent tissue, expressing normal levels of *Foxa1/2* genes, was inadvertently included and therefore

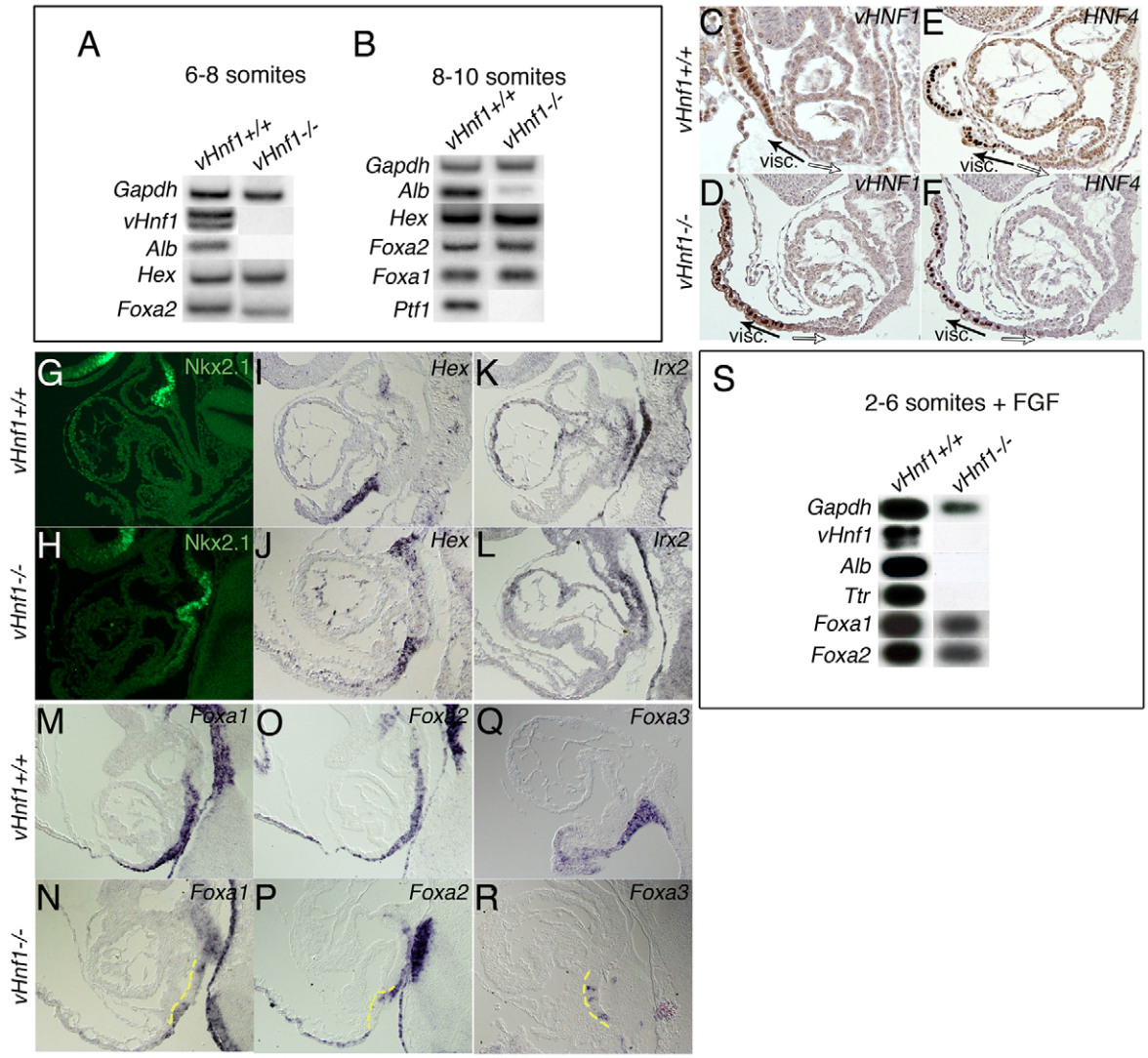

Fig. 5. vHNF1 is required for hepatic specification of the ventral endoderm. (**A**,**B**) Semi-quantitative RT-PCR analysis of RNA from WT and *vHnf1*$^{-/-}$ ventral endoderm isolated from 6-8s (A) or 8-10s (B) mouse embryos. *Gapdh* was used for normalization. *Hex*, *Foxa2* at 6-8s and *Hex*, *Foxa1*, *Foxa2* at 8-10s are expressed in the hepatic endoderm of *vHnf1*$^{-/-}$ mutant embryos. Defective hepatic and ventral pancreatic specification in the mutants is shown by the absence of *Alb* (A,B) and *Ptf1a* (B). (**C-F**) Immunostaining on sagittal sections of 8s control (*vHnf1*$^{+/+}$) (C,E) and *vHnf1*$^{-/-}$ (D,F) embryos using vHNF1 (C,D) and HNF4α (E,F) antibodies indicates a similar position of the extraembryonic visceral endoderm (visc.; black arrow; vHNF1- and HNF4α-positive) relative to the ventral endoderm (white arrow). Note that the vHNF1 antibody does not detect the weak levels of vHNF1 expression in the ventral endoderm as compared with the visceral endoderm (visc.; black arrow). (**G-R**) Immunostaining (G,H) and in situ hybridization (I-R) on sagittal sections of 8-10s control (G,I,K,M,O,Q) and *vHnf1*$^{-/-}$ (H,J,L,N,P,R) embryos using the lung marker *Nkx2.1* (G,H), the thyroid-hepatic marker *Hex* (I,J) and the lung-tracheopharyngeal marker *Irx2* (K,L) show apparently correct foregut endoderm regionalization in mutant embryos. Expression of *Foxa1* (M,N), *Foxa2* (O,P) and *Foxa3* (Q,R) is strongly downregulated in the prehepatic domain of mutant embryos (N,P,R, yellow dashed lines) as compared with WT (M,O,Q). (**S**) RT-PCR analysis of hepatic RNA from microdissected ventral endoderm of 2-5s control or *vHnf1*$^{-/-}$ embryos that has been cultured for 48 hours with bFGF plus heparan sulfate proteoglycan. Normal induction of hepatic specification, as indicated by *Alb* and *Ttr* expression in WT explants, is disrupted in the *vHnf1*$^{-/-}$ explant cultures.

biased our quantifications. More importantly, a further decrease in *Foxa1/2* expression was observed in 14s stage mutant ventral endoderm (see Fig. S2 in the supplementary material). Moreover, the induction of *Foxa3* expression was severely perturbed as indicated by rare *Foxa3*-positive cells distributed throughout the *vHnf1*$^{-/-}$ ventral endoderm (Fig. 5R). These data together clearly indicate a requirement of vHNF1 for maintaining the expression of *Foxa1* and *Foxa2* specifically in the ventral endoderm, as well as for *Foxa3* induction.

We then addressed the competence of *vHnf1*$^{-/-}$ endoderm to be specified into a hepatic fate. Ventral foregut endoderm was dissected from embryos at the 2-6s stage and cultured for 48 hours in the presence of bFGF (FGF2) and heparan sulfate proteoglycan, under conditions reported to be sufficient to induce hepatic specification in the absence of cardiac mesoderm (Calmont et al., 2006; Deutsch et al., 2001; Lee et al., 2005). Our experiments show that, in contrast to WT embryos, *vHnf1*$^{-/-}$ ventral endoderm (n=3 embryos) was not competent to induce the expression of *Alb* or *Ttr* (Fig. 5S). These

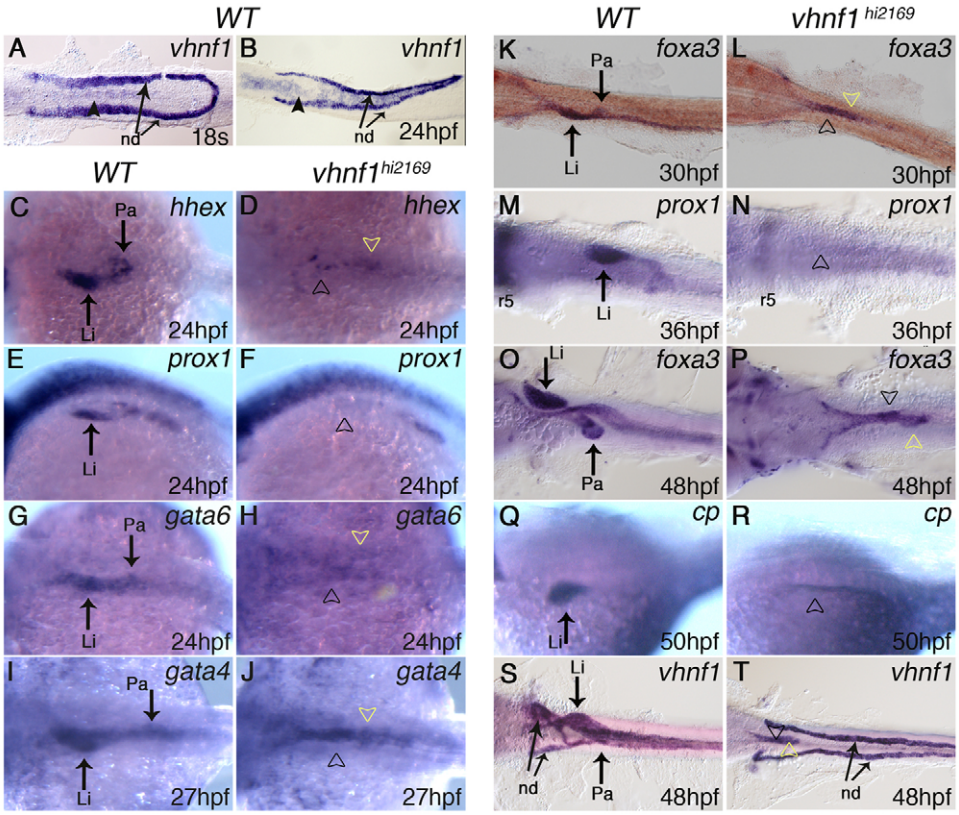

Fig. 6. vHNF1 is required for the formation of endoderm derivatives in zebrafish. (**A-T**) Whole-mount in situ hybridization in dorsal (A-D,G-L,Q-R), lateral (E,F), or ventral views (M-P,S-T) at 24-30 hpf (C-L) and 36-50 hpf (M-T). Anterior to the left. *krox20* (*egr2b*) *or frb35* (*egr2a*) staining was used to identify rhombomere 5 (r5), which is strongly reduced in *vhnf1*^{hi2169} homozygous embryos. (A,B) In WT zebrafish embryos, *vhnf1* is strongly expressed by 18s in the nephric duct (nd, arrows) and weakly in the gut endoderm (black arrowhead). *hhex* is expressed in the presumptive liver (Li) and pancreas (Pa) buds at 24 hpf (C). In *vhnf1*^{hi2169}, *hhex* expression is lost (arrowheads in D) and *prox1*, which is normally exclusively expressed in the liver (E,M), is absent (arrowheads in F,N). *gata6*, *gata4* and *foxa3* are normally expressed in the gut endoderm, liver and pancreas at 24-30 hpf (G,I,K) and 48 hpf (O). In *vhnf1*^{hi2169}, *gata6* expression is lost (arrowheads in H), whereas *gata4* (J) and *foxa3* (L,P) expression is maintained in the gut endoderm. Expression of the liver-specific marker *ceruloplasmin* (*cp*) (Q) is totally abolished in mutants (R). Analyses of *vhnf1* mutant transcripts show its expression in the nephric duct and in the mutant gut endoderm, but a complete absence of any endodermal budding (compare S with T).

data suggest that the failure of hepatic specification in the *vHnf1*$^{-/-}$ embryo is, at least in part, owing to a loss of competence to respond to FGF inductive signaling.

The role of vHNF1 in hepatic development is conserved between species

In zebrafish, the signaling molecules and the tissue interactions involved in early liver induction are less well documented than in chick or mouse. Hepatoblast specification in zebrafish is thought to occur at approximately 22 hours post-fertilization (hpf) as marked by the localized expression of *hhex* and *prox1*, which are among the earliest markers of liver development (Ober et al., 2003). Between 24 and 28 hpf, two thickened regions emerge from the intestinal rod, the anterior of which corresponds to the liver bud and the posterior to the pancreas bud (Field et al., 2003). Recent studies point to a conserved molecular program orchestrating liver formation in this organism. As in amniotes, FGF and BMP signaling pathways have been shown to be required for liver induction (Shin et al., 2007). Moreover, a role for Wnt signaling from the lateral plate mesoderm in zebrafish liver induction has been recently described (Ober et al., 2006). Additionally, in contrast to in amniotes, Hedgehog signaling appears to regulate the proliferation of already specified liver progenitors (Wallace and Pack, 2003). It has also been reported that *hhex* is required for liver development and normal gut looping in zebrafish (Wallace et al., 2001), whereas *gata4* and *gata6* play an important, yet redundant, role in liver expansion and differentiation (Holtzinger and Evans, 2005).

Several *vhnf1* zebrafish mutants presenting abnormal liver and pancreas development, in addition to defective hindbrain segmentation, have been isolated (Song et al., 2007; Sun and Hopkins, 2001). These mutants present a wide range of hepatic phenotypes from reduced liver and abnormal biliary development (Matthews et al., 2004; Sun and Hopkins, 2001) to the lack of a discernible liver at 3 dpf (Song et al., 2007). These observations prompted to us to examine in this organism the role of *vhnf1* at the stage of hepatic specification. For this analysis we chose the *vhnf1*^{hi2169} mutants because they are highly hypomorphic leading to null alleles (Bagnat et al., 2007; Sun and Hopkins, 2001).

Although the general expression pattern of *vhnf1* in zebrafish embryos has been reported previously (Song et al., 2007; Sun and Hopkins, 2001), its early expression in the endoderm before and during hepatic budding is less well documented. As shown in Fig. 6A,B, at both 18s and 22-24s stages, *vhnf1* was strongly expressed in the pronephric duct, while weakly expressed in the gut endoderm.

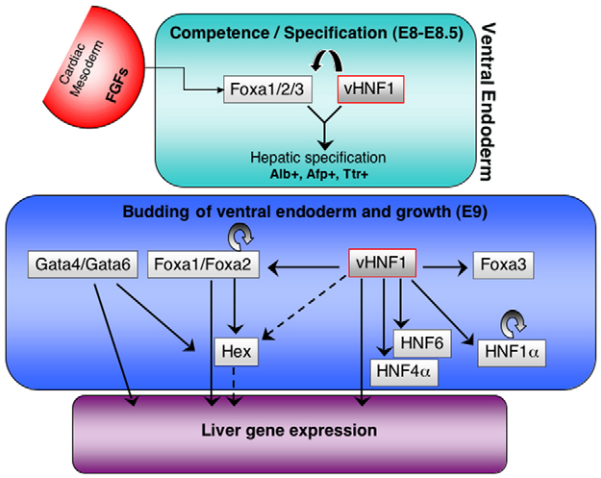

Fig. 7. Proposed model for vHNF1 function in liver induction. At the competence/specification step, FOXA1/2/3 and vHNF1 cooperate to confer responsiveness of the ventral endoderm to FGF signals and to induce the typically hepatic specification markers *Alb*, *Ttr* and *Afp*. In this process, *Foxa2* is directly downstream of FGFs (Zhang et al., 2004), whereas vHNF1 maintains Foxa factor expression and that of an as yet unidentified downstream target of the FGF pathway. In addition, GATA4/6 factors (not shown in this model) are directly induced by BMP2/4 and probably also by FGFs (Zaret, 2002) and have been proposed to mediate hepatic competence. At the budding step, a more complex regulatory network is established: FOXA2 induces *Hex* expression and is autoregulated, as HNF1α. vHNF1 has a preeminent role in this transcriptional circuit by inducing *Hnf4a*, *Hnf6* (Poll et al., 2006), *Hnf1a* (Kyrmizi et al., 2006), *Foxa3* (this study) (Hiemisch et al., 1997) and by maintaining *Foxa2* expression (Kyrmizi et al., 2006). This regulatory circuit becomes highly interconnected and integrates additional regulators at later stages (Kyrmizi et al., 2006) to sustain the expression of differentiated hepatic functions. Dashed arrows indicate epistatic relationships.

By 48 hpf, *vhnf1* was expressed in the gut and in the liver and pancreas anlagen (Fig. 6S), displaying an endoderm expression pattern similar to that of *foxa3* (Fig. 6O) (Field et al., 2003).

In situ hybridization analysis at the time of liver induction showed that the expression of both *hhex and prox1*, which at 24 hpf are normally expressed in the nascent liver and pancreas buds (Fig. 6C,E), was completely abrogated in these structures in the *vhnf1*^{hi2169} mutant (Fig. 6D,F). Unlike our observations in *vHnf1*$^{-/-}$ mouse embryos (Fig. 4), in zebrafish *vhnf1*^{hi2169} mutants expression of *gata6* was lost from the entire endoderm (Fig. 6H). By contrast, *gata4* expression at 27 hpf, or *foxa3* at 30 hpf, was maintained in the gut endoderm, but was not detected in the hepatic or pancreatic domain (Fig. 6J,L). Similar results were obtained at 30 and 48 hpf indicating that *vhnf1* mutants exhibit a profound defect in liver induction rather than a delay in its formation (Fig. 6M-P). As expected, expression of the specific liver marker *ceruloplasmin* (*cp*) was absent in mutant embryos (Fig. 6R). As *vhnf1* transcripts are produced from the *vhnf1*^{hi2169} mutant allele, we performed an in situ hybridization on 48 hpf mutant embryos using a full-length *vhnf1* probe and detected the presence of mutant transcripts only in the gut endoderm (Fig. 6T). This observation further confirms the complete absence of both the liver and pancreas buds. It also excludes the possibility that hepatoblasts or pancreatic cells, lacking the expression of key regulatory factors, were still formed in the absence of *vhnf1* function. Interestingly, the mutant gut endoderm did not exhibit the typical leftward bending observed in WT embryos (Fig. 6, compare S with T), and was abnormally regionalized as manifested by the caudal expansion of *foxa2* and *shh* expression (see Fig. S3 in the supplementary material) (Sun and Hopkins, 2001), indicating that *vhnf1* is also required for gut morphogenesis. Together, these data strongly suggest that *vhnf1*^{hi2169} mutant embryos exhibit defective hepatic specification, whereas the gut endoderm integrity appears to be maintained.

DISCUSSION

Our analysis of the function of the transcription factor vHNF1 reveals its essential role in the initiation of liver development in vertebrates. We show that vHNF1 is required for hepatic specification of the mouse ventral endoderm and for subsequent bud formation. A consequence of this defect is the absence of hepatoblast migration into the adjacent STM. Despite this, mutant liver lobes are correctly formed, although highly hypoplastic. In *Prox1* mutant mouse embryos, liver lobes are also correctly shaped despite a highly reduced number of hepatoblasts (Sosa-Pineda et al., 2000). Unlike *Prox1* mutants, in E13.5 *vHnf1*$^{-/-}$ embryos the liver architecture is severely disrupted and followed by massive apoptosis. Since *vHnf1* is not expressed in the STM, these observations imply that signals from hepatoblasts play a crucial role in the maintenance and generation of the hepatic architecture required for normal liver function.

Analysis at the time of liver induction indicates that vHNF1 is required for the acquisition of competence of the ventral endoderm to respond to inductive signals and for hepatic specification. Remarkably, the liver induction defect exhibited by *vHnf1*$^{-/-}$ embryos is basically identical to that reported in compound *Foxa1/Foxa2* mutants. In both cases, the ventral endoderm fails to acquire a hepatic fate when cultured in vitro under conditions reported to be sufficient to induce its specification. The acquisition of hepatic competence is disrupted even though *Foxa1* and *Foxa2* transcripts are present, albeit at lower levels, in *vHnf1*$^{-/-}$ ventral endoderm. Thus, vHNF1, like Foxa factors, mediates regional competence of the ventral endoderm to respond to FGF signaling. Because of their ability to open highly condensed chromatin structure upon binding, it has been proposed that FOXA and GATA factors behave as ‘pioneer factors’ that mark their target genes as competent to be expressed when exposed to the appropriate inductive signals (Cirillo et al., 2002). Given the structural properties of the protein it is unlikely that vHNF1 mediates competence by relieving chromatin condensation. One explanation that we explored was whether vHNF1 is a direct target of the FGF signaling pathway. Prior studies in zebrafish supported this hypothesis, as injected *vhnf1* mRNA can rescue the abnormal pancreatic and liver phenotype caused by the disruption of either FGF or BMP signaling pathways (Song et al., 2007), indicating that *vhnf1* is downstream of these pathways. Moreover, recent studies in mice have shown that hepatic gene induction is elicited by an FGF/MAPK pathway, whereas the FGF/PI3K pathway is required for growth and morphogenesis of the hepatic bud (Calmont et al., 2006). In *vHnf1*$^{-/-}$ mouse embryos, both processes are affected as hepatic specification is disrupted and the liver bud fails to form. However, when ventral endoderm was cultured under different conditions, we found that *vHnf1* expression is influenced neither by FGF signaling nor by pharmacological inhibitors of FGF signaling, indicating that vHNF1 is not a direct target of FGF signals. Moreover, *Fgfr2*, sprouty 2, *Dusp6* (*Mkp3*) and *Socs3* genes were induced at similar levels in *vHnf1*$^{-/-}$ and WT ventral endoderm

(see Fig. S4 in the supplementary material), suggesting that the hepatic competence defect of our mutants is not due to a global disruption of FGF signaling. Based on these observations, we propose that vHNF1 could mediate competence of the endoderm to respond to FGF signaling by controlling the expression of a downstream target of this pathway. An alternative, non-mutually exclusive possibility is that the reduced expression of *Foxa1* and *Foxa2*, along with the absence of *Foxa3*, lead to a level of Foxa proteins that is below the threshold required for relieving chromatin condensation and subsequent acquisition of the hepatic competence. Considering the broad expression of both *vHnf1* and *Foxa1/2* genes within the gut endoderm, it is difficult to explain how these factors, acting either in synergy or in a linear cascade, could mediate hepatic specification in a restricted domain of the ventral endoderm. It remains possible that local signals induce a hepatic specification factor(s) or co-factor(s) that either cooperates with vHNF1 or is an obligatory partner of this gene.

Following the competence step, vHNF1 appears to function through sequential and complementary mechanisms. One of them concerns the proper regional specification of the gut and the subsequent accurate acquisition of cell fates. Abnormal regional gut specification in *vHnf1*-deficient mutants at E9.0 is manifested by the caudal expansion of *Foxa2* and *Shh* expression, along with the ectopic expression of *Gata4* in the presumptive hepatic domain, which otherwise essentially lacks all early hepatic markers examined. One interesting possibility is that ectopic expression of *Gata4* reflects a change in the identity of the ventral endoderm into a duodenal-like fate. In agreement with the acquisition of a duodenal fate is the observation of sparse cells coexpressing PDX1 and GATA4 in the presumptive hepatic endoderm at E9. Since a ventral pancreas bud is not formed at any stage in *vHnf1* mutant embryos (Haumaitre et al., 2005), these PDX1-positive cells might represent duodenal precursors. A similar conversion of ventral pancreas progenitor cells into duodenal cells has been observed in mouse *Ptf1a* mutants (Kawaguchi et al., 2002). Interestingly, *Ptf1a* is not induced in *vHnf1*$^{-/-}$ ventral endoderm at any developmental stage (Fig. 5) (Haumaitre et al., 2005). Such cell-fate conversion would account, at least in part, for the abrogated morphogenesis and the lack of liver bud outgrowth.

Unlike the strongly downregulated expression of early hepatic genes at E9.0, at the 8-10s stage, *Foxa1*, *Foxa2* and *Hex* are expressed, albeit at different levels, in the presumptive hepatic domain of *vHnf1* mutants. Thus, vHNF1 is not required for the initial induction of these genes but rather for maintenance of their expression. These observations further suggest that vHNF1 and FOXA1 and FOXA2 factors do not act in a simple linear cascade that leads to the activation of liver-specific genes, but rather in synergistic pathways (Fig. 7) to activate these genes. This phase of induction of Foxa genes is shortly followed by a maintenance phase in which vHNF1 is required both for their sustained expression and for subsequent induction of other hepatic transcription factors, thus establishing a complex cross-regulatory network involved in the determination of the hepatic fate (Cereghini, 1996). Consistent with this model (Fig. 7), functional HNF1 (HNF1A – Mouse Genome Informatics) binding sites in the regulatory sequences of both *Foxa2* (Kyrmizi et al., 2006) and *Foxa3* (Hiemisch et al., 1997) have been identified. In addition, in silico analysis has indicated the presence of potential HNF1 binding sites within the upstream sequences of the *Foxa1* gene.

More importantly, a comprehensive analysis of promoter occupancy during mouse hepatic development from E14 has recently shown that vHNF1, but not the structurally related protein HNF1, is recruited to the promoter regions of a 'core' group of transcription factors including FOXA2, HNF1, HNF4 (HNF4A), HNF6 and GATA6, which compose autoregulatory and cross-regulatory circuits (Kyrmizi et al., 2006). Interestingly, the complexity and stability of this network increase gradually during organogenesis (Kyrmizi et al., 2006).

As in mice, in zebrafish *vhnf1* mutant embryos neither the liver nor the pancreas bud is formed and the gut endoderm is abnormally regionalized (see Fig. S3 in the supplementary material) (Sun and Hopkins, 2001). Despite the fact that in these two organisms the positioning of the gut endoderm relative to the adjacent tissues and the inductive signals are not the same (Ober et al., 2006), our analyses in zebrafish confirm an evolutionary conserved role of *vHnf1* in hepatic specification and development. Interestingly, the later roles of vHNF1 in intrahepatic biliary morphogenesis are also conserved in zebrafish (Matthews et al., 2004). Therefore, vHNF1 represents the earliest transcription factor involved in zebrafish hepatic specification.

Together, these studies support the notion that vHNF1 acts at two levels during vertebrate organogenesis. First, in the early acquisition of a hepatic and pancreatic fate from the multipotent ventral endoderm. Second, in the normal epithelial morphogenesis of tubular structures [i.e. biliary duct (Coffinier et al., 2002), stomach epithelium (Haumaitre et al., 2005) and gut lumen in zebrafish (Bagnat et al., 2007)]. Interestingly, these dual functions of vHNF1 correlate with its dynamic embryonic expression pattern, being initially high throughout the entire liver and pancreas buds and subsequently restricted to the branched pancreatic ductal network and the biliary system.

The strategies used to differentiate embryonic stem cells into endodermal cells are based on the knowledge of the conserved molecular network that controls endoderm formation in different vertebrate embryos (D'Amour et al., 2005). From a therapeutic point of view, it is important to now precisely define how the endoderm is patterned and how particular organs are induced. Our studies provide insights into the early molecular events of liver specification in vertebrates and may contribute to the development of in vitro strategies for the generation of hepatic cells for regenerative medicine, either from embryonic stem (Gouon-Evans et al., 2006) or somatic stem cells.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/16/2777/DC1

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