The homeobox gene *Hhex* is essential for proper hepatoblast differentiation and bile duct morphogenesis

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Abstract

Hhex is required for early development of the liver. A null mutation of *Hhex* results in a failure to form the liver bud and embryonic lethality. Therefore, *Hhex* null mice are not informative as to whether this gene is required during later stages of hepatobiliary morphogenesis. To address this question, we derived *Hhex* conditional null mice using the Cre-loxP system and two different Cre transgenics (*Foxa3-Cre*, and *Alfp-Cre*). Deletion of *Hhex* in the hepatic diverticulum (*Foxa3-Cre;Hhex^{d2,3/-}*) led to embryonic lethality, and resulted in a small and cystic liver with loss of *Hnf4α* and *Hnf6* expression in early hepatoblasts. In addition, the gall bladder was absent and the extrahepatic bile duct could not be identified. Loss of *Hhex* in the embryonic liver (*Alfp-Cre;Hhex^{d2,3/-}*) caused irregular development of intrahepatic bile ducts and an absence of *Hnf1β* in many (cystic) biliary epithelial cells, which resulted in a slow, progressive form of polycystic liver disease in adult mice. Thus, we have shown that *Hhex* is required during multiple stages of hepatobiliary development. The altered expression of *Hnf4α*, *Hnf6* and *Hnf1β* in *Hhex* conditional null mice suggests that *Hhex* is an essential component of the genetic networks regulating hepatoblast differentiation and intrahepatic bile duct morphogenesis.

Keywords

Hhex; Liver; Biliary tract; Extrahepatic bile duct; Polycystic liver disease

INTRODUCTION

The liver and biliary tract arise from the hepatic diverticulum, which develops as an out-pocketing of the ventral foregut endoderm around embryonic day 9.5 (E9.5) in mice (reviewed in Lemaigre and Zaret, 2004). For the most part, the cranial portion of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree while the caudal portion forms the extrahepatic biliary tract (Du Bois, 1963;Shiojiri, 1979). Early hepatoblasts in the diverticulum migrate into the surrounding mesenchyme and form the liver bud and cystic primordium (gall bladder precursor) by E10.5. Bipotential hepatoblasts within the liver bud eventually give rise to hepatocytes and biliary epithelial cells (BEC), which line the bile ducts (Shiojiri, 1979; reviewed in Shiojiri, 1997). BEC of the intrahepatic bile ducts (IHBD) originate from hepatoblasts surrounding branches of the portal vein within the embryonic liver (Shiojiri,

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1979;Shiojiri, 1984). Development of IHBD occurs through a series of distinct stages including differentiation of BEC around E13.5, formation of the ductal plate by E15.5, and ductal plate remodeling that ends shortly after birth (Clotman et al., 2002). The ducts of the extrahepatic biliary tract are also lined by BEC, although development of the intrahepatic and extrahepatic biliary systems occurs separately and apparently through different mechanisms. The extrahepatic biliary system forms by budding from the hepatic diverticulum in close association with the liver bud, and is composed of the gall bladder and several relatively large ducts, which converge to form the extrahepatic bile duct (EHBD) that connects the liver to the duodenum (Du Bois, 1963;Shiojiri, 1979).

The homeobox gene *Hhex* is expressed in the ventral foregut endoderm at E8.5 (Thomas et al., 1998;Bogue et al., 2000), and is required for very early development of the liver (Hallaq et al., 2004;Keng et al., 2000;Martinez-Barbera et al., 2000). In *Hhex$^{-/-}$* mouse embryos, the hepatic diverticulum is specified in the foregut endoderm, but proliferation of cells in the diverticulum is reduced (Bort et al., 2004) and subsequent migration of early hepatoblasts into the surrounding mesenchyme fails to occur (Bort et al., 2006). As a result, the liver bud is absent in *Hhex* null embryos (Keng et al., 2000;Martinez-Barberra et al., 2000). In addition, *Hhex* mutants are lacking the cystic primordium, which has been attributed to defective movements within the foregut endoderm (Bort et al., 2004). Therefore, *Hhex* is essential for the delamination and migration of early hepatoblasts from the hepatic diverticulum in formation of the liver bud, and in the absence of Hhex hepatic development is arrested, after the specification stage, at E9.5.

The expression pattern of *Hhex* has also been described in hepatobiliary tissue during later stages of development. *Hhex* mRNA has been detected in the liver bud, the embryonic liver, the gall bladder and the extrahepatic bile duct (Thomas et al., 1998;Bogue et al., 2000). Furthermore, *Hhex* is expressed in the adult liver in hepatocytes and IHBD cells (Bogue et al., 2000;Keng et al., 1998), and *Hhex* has been shown to regulate the in-vitro expression of the *ntcp* gene (Denson et al., 2000), which encodes a bile acid transporter expressed in adult hepatocytes. These findings suggest that *Hhex* has important roles during later stages of hepatobiliary morphogenesis, which thus far have not been addressed using genetic means because of the very early arrest in liver and biliary tract development in *Hhex$^{-/-}$* embryos.

In this study, we have developed a *Hhex* conditional null allele using the Cre-loxP system to determine the function of Hhex during later stages of liver and biliary tract development. We employed the *Foxa3-Cre* (Lee et al., 2005a;Lee et al., 2005b) and *Alfp-Cre* (Parviz et al., 2003) transgenes to eliminate *Hhex* in the hepatic endoderm at different developmental stages. Elimination of *Hhex* in the hepatic diverticulum (*Foxa3-Cre;Hhex$^{d2,3/-}$*) resulted in a severely hypoplastic and cystic liver, with major defects in development of the extrahepatic biliary tract. The liver-enriched transcription factors (LETF's) HNF4α and HNF6 were absent in hepatoblasts of *Foxa3-Cre;Hhex$^{d2,3/-}$* embryos at E13.5, which resulted in major defects in hepatic epithelial development. Deletion of *Hhex* in the embryonic liver (*Alfp-Cre;Hhex$^{d2,3/-}$*) caused abnormal morphogenesis of IHBD, with a down-regulation of *Hnf1β* in many biliary epithelial cells, and polycystic liver disease in adult mutant mice. Our results show that Hhex is required in hepatobiliary development after liver bud formation, and that Hhex is essential for proper hepatoblast differentiation and bile duct development.

MATERIALS AND METHODS

Derivation of *Hhex^{neoflox}* and *Hhex^{flox}* alleles

To construct the targeting vector, a *Hhex* P1 genomic clone was isolated (Bogue et al., 2003). A *loxP* element was inserted in between XbaI and SpeI sites of the 3rd intron of a *Hhex* 3' genomic fragment. An XhoI-BsaI 3' *Hhex* fragment containing this *loxP* site insertion was

blunt-end ligated into a BglII site in plasmid GR88 (from Trevor Williams, Univ. of Colorado Health Sciences Center). This plasmid contained a *loxP* site upstream of a *neomycin* resistance cassette flanked by FRT sites. Finally, a 3.8kb 5' *Hhex* SnaBI-XhoI fragment was blunt-end ligated onto the above 3' construct. The final targeting vector was sequenced in its entirety and used for the generation of *Hhex^{neoflox}* ES cell clones by the Animal Genomics Services at the Yale School of Medicine according to standard procedures (Nagy et al., 2002). Mice used in this study were maintained on mixed backgrounds (129S1/Sv and C57Bl/6).

The *Flp* transgenic mouse (Rodriquez et al., 2000) was provided by the Animal Genomics Services at the Yale School of Medicine. The *Hhex^{flox}* allele was cloned and sequenced to verify that the recombination event had occurred as designed.

Generation of *Hhex* conditional null mice

To derive our *Hhex* conditional null mice, *Alfp-Cre* (Parviz et al., 2003) and *Foxa3-Cre* (Lee et al., 2005a;2005b) mice were bred onto our *Hhex^{+/-}* background (Bogue et al., 2003). For almost all crosses, *Cre;Hhex^{+/-}* mice were bred to our *Hhex^{flox/+}* or *Hhex^{flox/flox}* mice. Embryos were staged according to Kaufman (1992). *Hhex^{+/-}* and *Hhex^{flox/-}* littermates were used as controls in comparison to *Hhex* conditional null mutants.

Histology and Immunohistochemistry

Embryos and adult liver tissue were processed for frozen and paraffin sectioning according to standard procedures (Nagy, et al., 2002). The antibodies used were as follows; rabbit anti-Hhex (Ghosh et al., 2000), rabbit anti-cytokeratin (DakoCytomation #Z0622), goat anti-HNF4α (Santa Cruz Biotechnology #SC-6556), rabbit anti-HNF6 (Santa Cruz Biotechnology #SC-13050), and goat anti-HNF1β (Santa Cruz Biotechnology #SC7411). Immunohistochemistry on sections was performed using Antigen Unmasking Solution (H-3300; Vector Labs), Avidin/Biotin Blocking kit (SP-2001; Vector Labs), Vectastain ABC kit (PK-6100; Vector Labs), and the DAB peroxidase substrate kit (SK-4100; Vector Labs) according to the manufacturer's protocols. For picro-sirius red staining, paraffin sections were cleared in xylene, rehydrated through an ethanol series, and rinsed in water. Slides were then placed in picro-sirius red solution (0.5 g Sirius red F3B in 500ml saturated aqueous picric acid; Sigma), for 1 hour at RT. Finally slides were washed twice in acetic acid water (5ml glacial acetic acid in 11ml distilled water), then dehydrated and cleared in xylene for mounting.

Immunofluorescence and Imaging

Immunofluorescence was performed according to standard procedures using Retrieve-All 2 (Signet Laboratories), Alexa Fluor 594 donkey anti-goat, Alexa Fluor 594 donkey anti-rabbit, and Alexa Fluor 488 donkey anti-rabbit (Molecular Probes), Hoechst 33342 (Molecular Probes), and the Prolong Antifade kit (P-7481, Molecular Probes) according to the manufacturer's recommendations. Antibodies used were as follows; anti-Ki-67 antibody (BD Pharmingen, #556003), anti-human cytokeratin (DakoCytomation #A0575), and anti-HNF4α (Santa Cruz #SC-6556). All fluorescent images were captured on a Zeiss Axiovert 200M with an Axiocam MRm camera using Axiovision software and the ApoTome mode. Images are a '3-D' representation of optical sections through approximately 3.5 microns of tissue.

RESULTS

Hhex is expressed in hepatobiliary tissue throughout development

We have previously detected *Hhex* mRNA in BEC of the extrahepatic and intrahepatic biliary tract during embryogenesis and in the adult liver (Bogue et al 2000). To extend these results, we analyzed *Hhex* expression in the liver and biliary tract by immunohistochemistry using a

polyclonal anti-Hhex antibody (Ghosh et al., 2000). At E10.5, Hhex is detected in hepatoblasts of the liver bud, and epithelial cells of the extrahepatic biliary tract precursors including the gall bladder primordia (Fig. 1A, B). At E15.5 Hhex was highly expressed in the extrahepatic bile duct (EHBD) epithelium (Fig. 1C) and in biliary cells of the ductal plate surrounding large branches of the portal vein near the liver hilum (Fig. 1D). Hhex was also detected in hepatocytes and biliary cells of the IHBD during late embryogenesis (Fig. 1E) and in the adult liver (Fig. 1F). Therefore, *Hhex* is expressed in the liver and biliary epithelium from the onset of hepatobiliary morphogenesis and into adulthood.

Derivation of a *Hhex* conditional null allele

To elucidate the role of *Hhex* in later stages of hepatobiliary development, we constructed a targeting vector to generate a *Hhex* conditional null allele using the Cre-loxP system (see Supplemental data, Fig. S1A). This construct was used to generate a targeted ES cell clone from which three chimeric founder males were obtained. Founder males were backcrossed to C57Bl/6 mice to derive the F1 generation of $Hhex^{neoflox/+}$ mice. Genotyping of the $Hhex^{neoflox/+}$ mice was performed by Southern and PCR (Supplementary Fig. S1B). $Hhex^{neoflox/+}$ F1 mice were then used to derive the *Hhex* conditional allele ($Hhex^{flox}$) after crossing to *Flp/Flp* transgenics (Rodriguez et al., 2000). This generated $Flp;Hhex^{flox/+}$ F1 mice, which were then backcrossed one additional generation to C57Bl/6. These $Hhex^{flox/+}$ F2 mice, and their progeny, were used for all further analysis, and genotyping was verified by Southern and PCR (Supplementary Fig. S1C). To confirm that the $Hhex^{flox}$ allele was functionally equivalent to the wild-type allele we intercrossed $Hhex^{flox/+}$ mice. $Hhex^{flox/flox}$ pups were born at a normal Mendalian ratio and were healthy and fertile, indicating that the $Hhex^{flox}$ allele was functionally wild-type. Finally, we used PCR and sequencing to confirm the presence of the $Hhex^{d2,3}$ allele in our *Hhex* conditional null mice after crossing the $Hhex^{flox}$ allele to *Alfp-Cre* mice (Supplementary Fig. S1D).

Hhex is essential for proper morphogenesis of the extrahepatic biliary tract

To test whether *Hhex* plays a role in the development of the extrahepatic biliary tract, we derived mutants in which *Hhex* was deleted in the hepatic diverticulum by crossing $Hhex^{flox}$ mice with the *Foxa3-Cre* transgene (Lee et al., 2005a) backcrossed to our $Hhex^{+/-}$ mice (Bogue et al., 1998). *Foxa3-Cre* has been shown to be active at E8.5 in ventral foregut endoderm, including the cells of the hepatic diverticulum (Lee et al., 2005a;Lee et al., 2005b). Thus, $Foxa3-Cre;Hhex^{d2,3/-}$ (conditional null) mice should lack *Hhex* both in the liver bud and extrahepatic biliary tract primordia.

In $Foxa3-Cre;Hhex^{d2,3/-}$ embryos at E10.5, Hhex was absent in almost all endoderm-derived cells of the liver bud and extrahepatic biliary tract primordia (compare Fig. 2A and B). Expression of Hhex was unaffected in endothelial cells within the $Foxa3-Cre;Hhex^{d2,3/-}$ liver confirming the deletion was specific to endoderm-derived cells (Fig. 2B). Therefore, *Hhex* was efficiently deleted in hepatobiliary tissue of mutant embryos by E10.5.

Development of the extrahepatic biliary tract was severely perturbed in $Foxa3-Cre;Hhex^{d2,3/-}$ embryos. The liver-enriched transcription factor HNF6 is normally detected in cells of the developing liver and in extrahepatic biliary tract precursors (Clotman et al., 2002;Pierreux et al., 2004). In control embryos at E10.5, HNF6 was expressed in the liver bud and gall bladder primordium (Fig. 2C) as previously described. However, the gall bladder primordium could not be identified in $Foxa3-Cre;Hhex^{d2,3/-}$ embryos (Fig. 2D). In addition, few cells were HNF6-positive in the liver bud of mutant animals at this stage. At E13.5, the $Foxa3-Cre;Hhex^{d2,3/-}$ mutant liver was hypoplastic compared to the control (compare Fig. 2E and F). Cytokeratin (CK) immunostaining revealed that development of the EHBD was also abnormal in $Foxa3-Cre;Hhex^{d2,3/-}$ embryos. At E13.5 in controls, the EHBD forms just caudal

to the liver and ventral to the main portal vein (Fig. 2G) and is lined by a single layer of columnar to cuboidal epithelial cells (Fig. 2I). In contrast, the EHBD appeared to be absent in *Foxa3-Cre;Hhex^{d2,3/-}* embryos (Fig. 2H), and the normal EHBD epithelium was replaced by pseudostratified epithelium resembling the duodenum (Fig. 2J). We verified that Hhex was absent in the abnormal, duct-like structure in the mutant by Hhex immunostaining (Fig. 2K,L).

Next we examined *Foxa3-Cre;Hhex^{d2,3/-}* embryos at E16.5 to see how the EHBD phenotype progressed. At E16.5, the EHBD forms a prominent duct that passes ventrolaterally from the liver hilum to the duodenum adjacent to the portal vein and pancreas, and is lined by a single cell layer of columnar epithelium (Fig. 3A, C). In *Foxa3-Cre;Hhex^{d2,3/-}* embryos, duodenal-like mucosa extended directly from the duodenum to the liver hilum (Fig. 3B, D). In addition, at the liver hilum, there appeared to be dilated hepatic ducts and biliary cysts lined by cuboidal epithelium (Fig. 3B). To confirm that this ectopic tissue was indeed duodenal epithelium, we performed HNF4α immunostaining. *Hnf4α* is expressed in the small intestine (Drewes et al., 1996), but not in BEC (Clotman et al., 2005). In control embryos, HNF4α was strongly expressed in the nucleus of epithelial cells lining the duodenum (Fig. 3E, G), with a very low level of expression in the epithelium lining the EHBD (Fig. 3G). In *Foxa3-Cre;Hhex^{d2,3/-}* embryos, HNF4α was strongly expressed in the ectopic duodenal-like epithelial cells (Fig. 3F), with expression localized to the nucleus in these cells up to the liver hilum (Fig. 3H). This result indicated that ectopic duodenal tissue apparently replaced the EHBD in *Foxa3-Cre;Hhex^{d2,3/-}* mutants.

In *Foxa3-Cre;Hhex^{d2,3/-}* embryos, single cell layer columnar/cuboidal epithelia was detected where the duodenum fused to the liver, suggesting the presence of biliary epithelia. Therefore, we examined the expression of HNF6, which is expressed in biliary epithelia but not duodenal epithelia (Fig. 3I). In *Foxa3-Cre;Hhex^{d2,3/-}* embryos, almost all of the ectopic duodenal tissue did not express HNF6, but some epithelial cells near the base of the liver were positive for HNF6 (Fig. 3J). These HNF6 positive cells most likely represent either hepatic duct epithelium or a remnant of the EHBD. We conclude that in *Foxa3-Cre;Hhex^{d2,3/-}* embryos, the EHBD is mostly or completely replaced by duodenum.

Hhex is required for normal hepatoblast differentiation

Deletion of *Hhex* in the hepatic diverticulum was embryonic lethal and resulted in a severely hypoplastic liver in all *Foxa3-Cre;Hhex^{d2,3/-}* mutants analyzed. We examined expression of *Hnf4α* (Fig. 4A-D), which is a marker for the hepatoblast in the early embryonic liver and is required for normal differentiation of hepatocytes (Parviz et al., 2003;Battle et al., 2006). In control embryos at E13.5, HNF4α was detected in hepatoblasts as expected (Fig. 4A, C). However, in *Foxa3-Cre;Hhex^{d2,3/-}* embryos, HNF4α was absent from almost all hepatic cells (Fig. 4B, D). A similar result was seen for the Onecut transcription factor HNF6 (Fig. 4E-H), which is required for proper hepatoblast differentiation and bile duct development (Clotman et al., 2002). Interestingly, by E16.5 HNF4α and HNF6 were detected in many parenchymal cells in the liver of *Foxa3-Cre;Hhex^{d2,3/-}* embryos, although a large number of hepatic cells were still negative (data not shown). These results suggest that Hhex is required for the expression of *Hnf4α* and *Hnf6* only during the early stages of hepatogenesis, and that loss of expression of these LETF's in early hepatoblasts of *Foxa3-Cre;Hhex^{d2,3/-}* mutants may lead to a disruption of normal hepatic architecture later in development.

By E16.5-E18.5, *Foxa3-Cre;Hhex^{d2,3/-}* livers were grossly abnormal compared to controls. At E16.5, in the mutant, tissue at the ventral liver periphery had lost its cohesive structure and this resulted in a trapping or pooling of blood cells in this region (compare Fig. 5A and B). At E18.5, normal hepatic tissue morphology is characterized by numerous portal vein branches associated with IHBD (Fig. 5C). However, in severely affected mutants, normal hepatic architecture was disrupted and mutant livers were extremely cystic with large dilated duct-like

structures and many smaller cysts (Fig. 5D). We examined these duct-like structures and cysts in greater detail to determine their cellular origin. In controls, immunostaining with a biliary-specific CK antibody showed that only BEC were strongly positive for CK (Fig. 5E). In *Foxa3-Cre;Hhex$^{d2,3/-}$* mutant embryos, many duct-like structures/cysts stained positive for biliary CK (Fig. 5F). The larger CK-positive duct-like structures probably represent dilated hepatic ducts, and other structures most likely represent dilated IHBD or biliary cysts. However, some duct-like structures/cysts had a heterogeneous epithelium with both CK-positive and CK-negative cells, whereas other cysts were composed entirely of cells that were CK-negative. To confirm that the heterogeneous cystic epithelium in mutants was not due to incomplete deletion of *Hhex* in these cells, we performed Hhex immunostaining at E18.5 (Fig. 5G, H). In *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants, Hhex was absent in parenchymal cells and cystic epithelial cells in the liver. HNF1β is a LETF expressed in biliary epithelia (Fig. 5I) and required in IHBD morphogenesis (Coffinier et al., 2002). However, in *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants, HNF1β was absent in many cystic biliary epithelial cells (Fig. 5J). Dual immunolabeling for CK and HNF4α confirmed that IHBD cells are normally CK-positive and HNF4α-negative (Fig. 5K). In contrast, some duct-like structures/cysts in *Foxa3-Cre;Hhex$^{d2,3/-}$* livers contained both CK-positive cells and HNF4α-positive cells (Fig. 5L). Although there was variability in the severity of cystogenesis in mutant embryos, mildly affected mutants also displayed a similarly abnormal hepatic epithelia as severely affected embryos. Some 'hybrid' cells that were both CK-positive and HNF4α-positive were detected and many cysts formed in the absence of a branch of the portal vein, suggesting the normal mechanism of BEC induction is not properly functioning in this mutant (Fig. 5M). In mutants, hepatocytes remained small and tightly clustered, suggesting a failure to differentiate normally. Therefore, we conclude that the absence of Hhex in the *Foxa3-Cre;Hhex$^{d2,3/-}$* liver primordium results in abnormal hepatoblast differentiation and severely abnormal hepatic architecture by late embryogenesis, most likely due to the loss of HNF4α and HNF6 expression in the early embryonic liver.

Hhex is required for proper development of the intrahepatic bile ducts

To determine if *Hhex* has an essential role in liver development during late embryogenesis, we used *Alfp-Cre* transgenic mice (Zhang et al., 2005;Krupczak-Hollis et al., 2004;Parviz et al., 2003). Previous studies have shown that *Alfp-Cre* provides deletion of a floxed allele in the liver by E15 (Krupczak-Hollis et al., 2004;Parviz et al., 2003). We generated *Alfp-Cre;Hhex$^{d2,3/-}$* (conditional null) mice by crossing our *Hhex^{flox}* mice to *Alfp-Cre;Hhex$^{+/-}$* mice.

To assess the extent of deletion of *Hhex* in *Alfp-Cre;Hhex$^{d2,3/-}$* livers, we performed Hhex immunohistochemistry. In controls at E18.5, Hhex was detected in the liver in hepatocytes, IHBD, and endothelial cells (Fig. 6A). In *Alfp-Cre;Hhex$^{d2,3/-}$* embryos, Hhex was absent in hepatocytes and biliary cells as expected (Fig. 6B). Deletion of *Hhex* in the *Alfp-Cre;Hhex$^{d2,3/-}$* liver was also confirmed by real-time RT-PCR (data not shown).

We examined *Alfp-Cre;Hhex$^{d2,3/-}$* livers at E18.5 for abnormal epithelial morphology. Hematoxylin and eosin staining showed that portal vein branches in mutants were surrounded by abnormal duct-like structures/cysts (compare Fig. 6C and D). Staining with the biliary CK antibody showed that control portal tracts had a normal pattern of a few scattered CK-positive cells around the portal vein, with one or two IHBD precursors (Fig. 6E). However, portal tracts in *Alfp-Cre;Hhex$^{d2,3/-}$* livers were often surrounded by many irregular biliary duct-like structures/cysts (Fig. 6F). HNF1β immunostaining of *Alfp-Cre;Hhex$^{d2,3/-}$* livers showed that, in severely affected mutants, this transcription factor was absent in many cystic biliary epithelial cells (compare Fig. 6G and H). Also, hybrid cells (both HNF4α- and CK-positive) were present in duct-like structures/cysts surrounding portal veins, and these hybrid cells were not detected in control IHBD (compare Fig. 6I and J). Finally, in mutants, many of the cysts present throughout the parenchymal tissue appeared to form in the absence of a branch of the

portal vein (compare Fig. 6K and L). The irregular duct-like structures/cysts in *Alfp-Cre;Hhex^{d2,3/-}* mutants is characteristic of a ductal plate malformation (DPM) and this suggests that *Hhex* plays a role in ductal plate remodeling and/or in the regulation of periportal cell differentiation.

Deletion of *Hhex* in the embryonic liver results in a slow, progressive form of polycystic liver disease

In contrast to deletion of *Hhex* in the hepatic diverticulum in *Foxa3-Cre;Hhex^{d2,3/-}* embryos, which was embryonic lethal, deletion of *Hhex* in the embryonic liver in *Alfp-Cre;Hhex^{d2,3/-}* mice was not lethal. Genotyping showed that in crosses between *Hhex^{flox/+}* mice and *Alfp-Cre;Hhex^{+/-}* mice, 14 out of 89 pups were *Alfp-Cre;Hhex^{d2,3/-}* (16% actual, 12.5% expected).

We examined livers in mice from 8 weeks to 73 weeks of age. Hepatic cysts were visible on the surface of the liver in 24 out of 26 *Alfp-Cre;Hhex^{d2,3/-}* mice, while none of the control mice examined had cysts (Fig. 7A-F). Analysis of four 8 week old mice revealed that the most severely affected liver had >50 visible hepatic cysts. The three other *Alfp-Cre;Hhex^{d2,3/-}* livers that were examined at this age had >29, >25, and >16 visible hepatic cysts. Many cysts were multiloculated and almost all cysts contained a clear fluid (Fig. 7B). Hepatic cysts visible at the liver surface ranged in size from approximately 0.5mm to 8mm in diameter. The largest cyst identified was approximately 8mm × 8mm × 15mm (Fig. 7D). Cysts were seen in all lobes of the liver, and were common near the ventral midline and periphery (Fig. 7F). There was some variability in the severity of the liver phenotypes among the different age groups. However, the severity of cystogenesis tended to be worse in older mutant animals. Most mutant mice examined around 1 year of age and older had severe polycystic liver disease (Fig. 7G, H).

In human polycystic liver disease, hepatic cysts arise from BEC and are associated with defects in the development of IHBD. Histological analysis of adult *Alfp-Cre;Hhex^{d2,3/-}* livers revealed severe abnormalities in IHBD morphogenesis. Normally, only one or two IHBD form near each branch of the portal vein (Fig. 8A). In *Alfp-Cre;Hhex^{d2,3/-}* livers, portal veins were surrounded by multiple cysts and irregular duct-like structures (Fig. 8B), most lined by a single layer of cuboidal or flattened epithelium (data not shown), suggesting that the cysts were of biliary cell origin. In addition, no red blood cells were detected in cysts examined by H+E staining at 8 weeks of age (data not shown), confirming that these cysts were not vascular structures.

To verify that the cysts found in *Alfp-Cre;Hhex^{d2,3/-}* livers were biliary in origin, we performed immunohistochemistry using the biliary CK antibody. In control livers, CK staining is confined to cells lining the IHBD (Fig. 8C), and is absent in periportal hepatocytes. In *Alfp-Cre;Hhex^{d2,3/-}* livers, portal veins were often surrounded by large CK-positive biliary cysts, and many smaller CK positive cysts, ducts, or ductal plate remnants (Fig. 8D). The abnormal expression of biliary-specific CK was also detected in some periportal cells. To examine these cysts further, we performed a histological stain for collagen. Bile ducts and portal veins are normally incorporated into a collagen-rich extracellular matrix. A relatively thin layer of collagen positive periportal mesenchyme surrounded portal veins and IHBD in controls (Fig. 8E). In severely affected *Alfp-Cre;Hhex^{d2,3/-}* livers, portal tracts and cysts were surrounded by an extensive layer of collagen deposition (Fig. 8F). From these results, we conclude that hepatic cysts in *Alfp-Cre;Hhex^{d2,3/-}* mice are lined by biliary cells and are a result of abnormal morphogenesis of IHBD.

In polycystic disease, cystic epithelial cells have an increased rate of proliferation when compared to normal epithelia (Gregoire et al., 1987; reviewed in Alvaro et al., 2006). In fact, normal biliary cells of IHBD have been described as postmitotic (Fabris et al., 2000). We

examined control bile ducts and *Alfp-Cre;Hhex$^{d2,3/-}$* cystic epithelia by dual-label immunofluorescence for the expression of the proliferation marker Ki-67 and biliary CK. In control mice, we found that only 2.3% (n=395) of CK-positive cells were also positive for Ki-67 (Fig. 8G-I). However, cystic biliary epithelia in mutant mice had a greater than four-fold higher rate of proliferation (9.6%, n=707; Fig. 8J-L) than normal biliary cells and this was a statistically significant difference (Student's T-test: $P < 0.05$). Therefore, abnormal proliferation of cystic biliary epithelial cells in *Alfp-Cre;Hhex$^{d2,3/-}$* mutants may contribute to the progression of cystogenesis in these mice.

DISCUSSION

Hhex has previously been shown to be essential in formation of the liver primordium in mouse. In *Hhex$^{-/-}$* mutant embryos, liver specification occurs but the liver bud fails to form (Keng et al., 2000;Martinez-Barbera et al., 2000). *Hhex* appears to be important for early ventral endodermal proliferation because in *Hhex$^{-/-}$* embryos, endodermal cells of the ventral-lateral foregut have a reduced rate of proliferation (Bort et al., 2004). As well, cells of the hepatic diverticulum in *Hhex$^{-/-}$* embryos fail to breakdown laminin and delaminate into the surrounding mesenchyme and this is associated with a defect in interkinetic nuclear migration and a failure in the conversion of the hepatic endoderm to a pseudostratified epithelium (Bort et al., 2006). *Hhex* is also required for budding of the cystic primordium and ventral pancreas (Bort et al., 2004;Bort et al., 2006). We have shown here that *Hhex* is expressed in hepatobiliary tissue throughout embryogenesis and in the adult. To overcome the very early arrest in hepatobiliary development in the *Hhex$^{-/-}$* embryos, we have derived *Hhex* conditional null mice using the Cre-loxP system, allowing us to address the roles of Hhex in later stages of liver and biliary development. We have shown, for the first time, that Hhex is required for hepatic differentiation and morphogenesis after liver bud formation. A summary of the differences between the *Hhex$^{-/-}$*, *Foxa3-Cre;Hhex$^{d2,3/-}$*, and *Alfp-Cre;Hhex$^{d2,3/-}$* mutant phenotypes is shown in Figure 9.

Hhex is necessary for the proper morphogenesis of the extrahepatic biliary tract

Hhex is expressed in the epithelium of the extrahepatic biliary tract, including the EHBD and gall bladder, from E10.5 through late embryogenesis. Deletion of *Hhex* in the hepatic diverticulum of *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants resulted in the absence of the gall bladder and replacement of the EHBD by duodenum. In some *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants there was a large ventral midline cystic structure, which is most likely derived from cystic duct epithelium, rather than gall bladder epithelium, because we could not identify the gall bladder primordium at E10.5 by HNF6 immunostaining. In no case was a well-defined gall bladder ever found in *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants at E18.5. The replacement of the EHBD by duodenum may result from an arrest in development, or a change in cell fate. Interestingly, it was recently suggested that *Hhex* has an important role in the epithelial transformation of ventral foregut endoderm cells (Bort et al., 2006). We speculate that, in the absence of Hhex, most cells in the caudal hepatic diverticulum that are normally fated to form the EHBD instead become duodenal cells. This hypothesis is supported by several lines of evidence. In *Hhex$^{-/-}$* mutant embryos, it has been suggested that cells of the hepatic diverticulum take on characteristics of duodenal cells at E10.5 because they ectopically express *gata4* and *shh* (Bort et al., 2006). In addition, the EHBD, duodenum, and pancreas normally develop from adjacent regions of the foregut endoderm, and it has previously been shown that this part of the endoderm retains some plasticity during development (Fukuda et al., 2006;Sumazaki et al., 2004;Offield et al., 1996). However, cell lineage analysis will be necessary to confirm a change in cell fate of hepatic endoderm cells in *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants. The observation of HNF6-positive epithelial cells at the base of the mutant liver, and in direct contact with duodenal epithelium, could suggest a remnant of the proximal EHBD. Therefore, Hhex may normally function to

suppress the duodenal differentiation program in the cells that give rise to more distal portions of the EHBD. It is interesting to note that the role of *Hhex* in EHBD development appears to be conserved, because in a *Hhex* morphant in zebrafish the EHBD could not be identified (Wallace et al., 2001).

Hhex is required in the early embryonic liver for normal hepatoblast differentiation

Our work strongly suggests that *Hhex* is essential in the proper differentiation of hepatoblasts. In *Foxa3-Cre;Hhex^d2,3/-* embryos, HNF4α and HNF6 were absent in hepatoblasts of the early embryonic liver. *Hnf4α* has been shown to be essential in hepatoblast differentiation and the establishment of normal hepatic parenchymal epithelium (Parviz et al., 2003). Conditional deletion of *Hnf4α* resulted in some livers that lost their cohesive structure, had large lesions, and were falling apart. Recently, HNF4α has been shown to regulate the expression of multiple proteins required in cell junction assembly and adhesion during liver development (Battle et al., 2006). In *Hnf6^-/-* mutants, differentiation of hepatoblasts was abnormal with excessive formation of biliary epithelial cells and cysts (Clotman et al., 2002). The absence of HNF4α and HNF6 in the early liver of *Foxa3-Cre;Hhex^d2,3/-* mutants most likely explains the severe defects in the hepatic epithelium seen later in development. These defects included a loss of cohesive structure in the ventral liver periphery, severe cystogenesis, and the presence of cystic epithelium with both biliary-like (CK-positive) and hepatocyte-like (HNF4α-positive) cells. *Hnf4α* and *Hnf6* are components of a complex genetic network of liver-enriched transcription factors, and how Hhex fits into this network remains to be determined. We suggest that Hhex has an essential role in hepatoblast differentiation in the early embryonic liver through positive regulation of *Hnf4α* and *Hnf6* expression. Why Hhex is absolutely essential for HNF4α and HNF6 expression only in the early hepatoblast is unknown, but other LETF's, such as Foxa2 (HNF3β) and HNF4α, have also been shown to function differently in the fetal versus the adult liver (Sund et al., 2000;Hayhurst et al., 2001). Interestingly, HNF6 has been shown to regulate the expression of *Hnf4α* in the embryonic liver (Braincot et al., 2004). The regulation of *Hnf4α* expression is complex with two promoters, P1 and P2, and their respective transcripts *Hnf4α1* and *Hnf4α7* (Briancot et al., 2004; Odom et al., 2004). HNF6 activates the P2 promoter in the embryonic liver, and throughout development there is a switch to almost exclusively P1 promoter activity in mature hepatocytes (Briancot et al., 2004). We have identified two conserved Hhex consensus binding sites approximately 15kb upstream of the *Hnf6* transcription start site (data not shown) suggesting that *Hnf6* may be a direct target of Hhex.

Hhex is essential during late embryogenesis for proper development of intrahepatic bile ducts

Deletion of *Hhex* in the liver during late embryogenesis in *Alfp-Cre;Hhex^d2,3/-* mutants resulted in abnormal morphogenesis of IHBD. In the mutant livers, portal tracts were surrounded by biliary cysts and irregular duct-like structures. Further, many periportal cells lining the cysts lacked HNF1β expression. *Hnf1β* has been shown to be essential in normal differentiation and development of IHBD in mice (Coffinier et al., 2002). In mutants with a targeted deletion of *Hnf1β* in the embryonic liver, the ductal plate was very disorganized with irregular duct-like structures at E17.5. We believe the absence of HNF1β in some cystic epithelial cells most likely reflects abnormal periportal cell differentiation. In support of this, we have shown that some cystic biliary cells were positive for HNF4α. These abnormal hybrid cells expressed both hepatoblast/hepatocyte (HNF4α) and biliary (CK) cell markers, and have previously been described in the *Hnf6^-/-* mutant (Clotman et al., 2005). HNF6 is a member of the Onecut family of transcription factors and was shown to be required for IHBD morphogenesis by regulation of hepatoblast differentiation (Clotman et al., 2002). The presence of hybrid cells in *Alfp-Cre;Hhex^d2,3/-* mutants suggests that Hhex is required for proper differentiation of hepatoblasts into hepatocytes and biliary cells. Perhaps in the absence of Hhex, some hepatoblasts arrest

their differentiation or certain hepatoblasts/hepatocytes may abnormally activate biliary cell differentiation.

Additionally, we have detected biliary cysts that were present throughout the parenchymal tissue (i.e. in the absence of a branch of the portal vein), which implies the normal mechanism of biliary cell induction in hepatoblasts adjacent to the mesenchyme of the portal vein is not functioning in these mutants. Similar results were seen in the *Hnf6$^{-/-}$;Oc-2$^{-/-}$* double knockout mice and it was suggested that these Onecut transcription factors control cell lineage decision by regulating periportal TGFβ signaling (Clotman et al., 2005). We did not detect any obvious differences in *Hnf6* expression during late embryogenesis between *Alfp-Cre;Hhex$^{d2,3/-}$* mutants and controls by immunohistochemistry (data not shown). Further studies are necessary to determine whether Hhex has a similar role in biliary cell differentiation as HNF6 by regulating the expression of components of the TGFβ pathway.

The liver phenotype in our *Alfp-Cre;Hhex$^{d2,3/-}$* mice resembles human polycystic liver disease. The most common forms of this disease involve a slow, progressive cystogenesis that can result in organ failure (reviewed in Tahvanainen et al., 2005). In most cases, the disease usually presents in the fourth or fifth decade of life, and liver function tests are often normal (Arnold and Harrison, 2005). Blood serum levels of total bilirubin, albumin, alanine transferase, and total protein from *Alfp-Cre;Hhex$^{d2,3/-}$* mice at 2 months of age were normal, but alkaline phosphatase was increased two-fold in severely affected mutants (data not shown). Polycystic liver disease is also associated with abnormal proliferation of the cystic epithelia (reviewed in Alvaro et al., 2006). In concurrence with this, *Alfp-Cre;Hhex$^{d2,3/-}$* mutants have cystic epithelia with greater than a four-fold increase in proliferation compared to control biliary cells. Another characteristic abnormality in all forms of polycystic disease of the liver is ductal plate malformation (DPM), which is defined as an arrest in embryonic morphogenesis of the ductal plate (reviewed by Desmet, 2004). Our findings of DPM in *Alfp-Cre;Hhex$^{d2,3/-}$* mutants indicate a failure in remodeling of the ductal plate and suggest *Hhex* may have a role in this process during embryogenesis.

The differences seen in the severity of the liver phenotypes between the *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants and the *Alfp-Cre;Hhex$^{d2,3/-}$* mutants can be explained by the difference in the timing of *Hhex* deletion (summarized in Figure 9). In *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants, deletion of *Hhex* appeared to be complete in the hepatobiliary primordia by E10.5, and this resulted in an absence of HNF4α and HNF6 expression in the liver at E13.5. Whereas in *Alfp-Cre;Hhex$^{d2,3/-}$* mutants, deletion of *Hhex* was restricted to endodermal cells of the liver and did not appear to be complete in most mutants until late embryogenesis with no affect on HNF4α or HNF6 expression in the early liver. Our findings regarding the timing of deletion of a floxed allele using these Cre transgenics are similar to previous studies (Parviz et al., 2003;Lee et al., 2005a;Lee et al., 2005b). The difference between the *Hhex$^{-/-}$* mutant and the *Foxa3-Cre;Hhex$^{d2,3/-}$* conditional mutant is probably due to strain-specific differences between these mice and/or perdurance of Hhex protein in the conditional mutant in early hepatoblasts migrating from the hepatic diverticulum to form the liver bud.

It is interesting to note here that *Hhex* has been shown to play a crucial role in several cell-lineage differentiation pathways including hematopoiesis, lymphopoiesis, cardiogenesis, and vascular smooth muscle cell differentiation (Kubo et al., 2005;Bogue et al., 2003;Foley and Mercola, 2005;Oyama et al., 2004). Our results suggest that Hhex has a similar role in regulating hepatoblast differentiation into hepatocytes and biliary cells. In support of this hypothesis, liver regeneration studies in rat have shown that *Hhex* expression is upregulated after partial hepatectomy (Tanaka et al., 1999). We are currently searching for Hhex target genes involved in these cell fate decisions.

Conclusions

We have shown for the first time that *Hhex* has distinct functions during different stages of liver and biliary tract development. Our work has demonstrated essential roles for *Hhex* in hepatoblast differentiation and morphogenesis of the bile ducts. Future studies on the Hhex gene in hepatocyte and biliary cell differentiation should lead to novel insights into hepatobiliary disorders in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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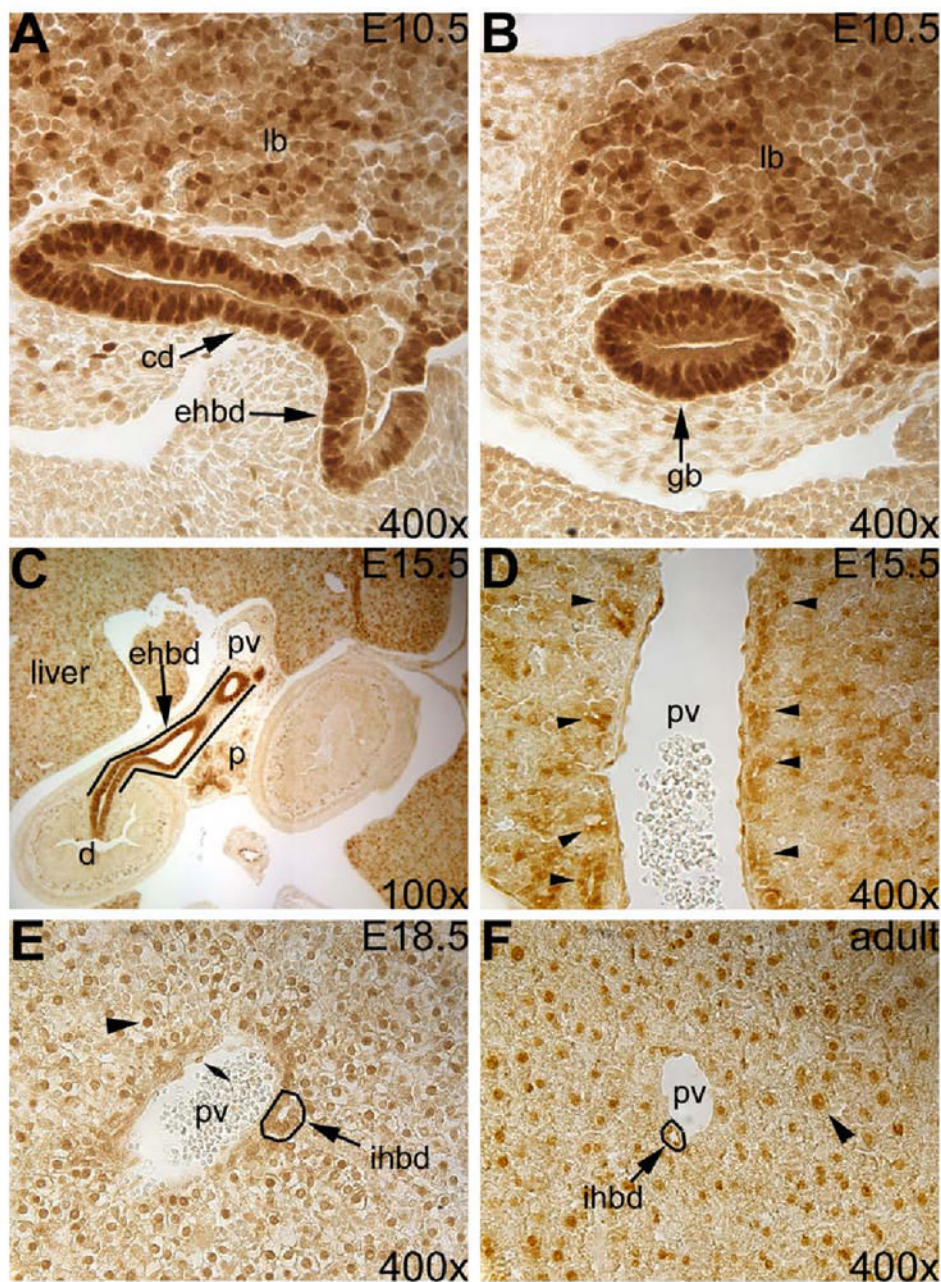
Fig 1. Hhex immunostaining reveals expression in the liver and biliary epithelium throughout embryogenesis and in the adult

(A) E10.5 *Hhex* expression in the liver bud (lb), the extrahepatic bile duct precursor (ehbd) and the cystic duct precursor (cd). (B) Hhex is detected in the gall bladder primordium (gb) at E10.5. (C) *Hhex* expression in the EHBD (outlined) at E15.5. (D) Hhex is also expressed in the biliary cells of the ductal plate (arrowheads) near the liver hilum. (E) Hhex is present in hepatocytes (arrowhead), intrahepatic bile duct cells (arrow), and endothelial cells (double arrowhead) at E18.5. (F) In the adult liver, Hhex is detected in hepatocytes (arrowhead) and biliary cells (arrow). ihbd=intrahepatic bile duct, d=duodenum, p=pancreas, pv=portal vein.
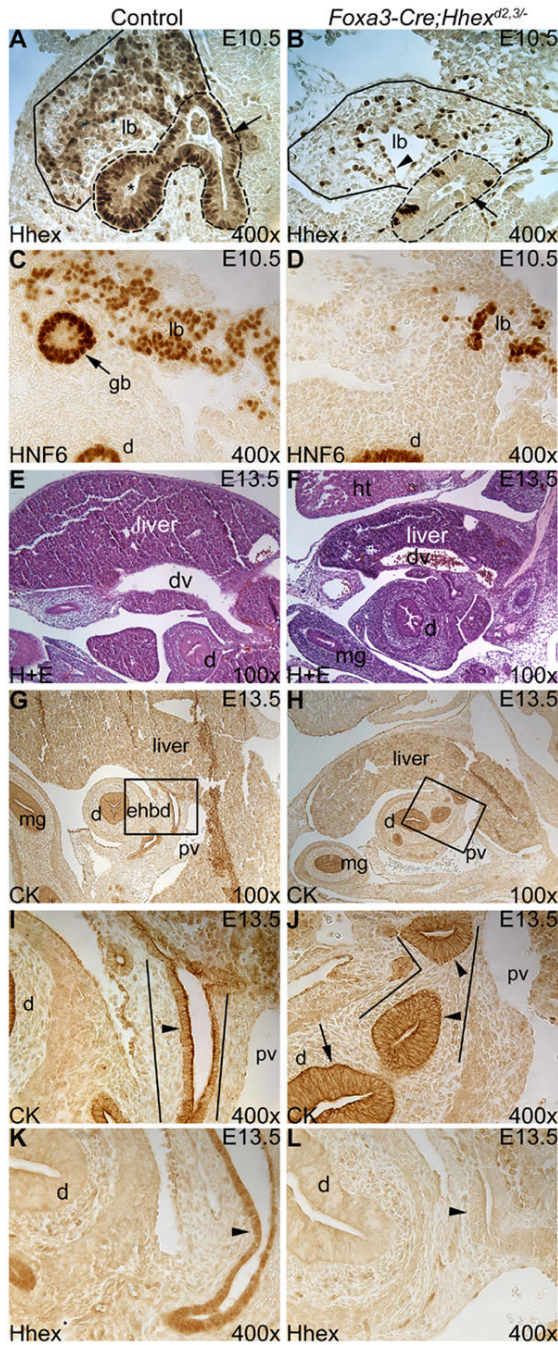
Fig 2. Deletion of *Hhex* in the hepatic diverticulum in *Foxa3-Cre;Hhex*$^{d2,3/-}$ mutant embryos results in severe abnormalities in early development of the extrahepatic biliary tract
(A,B) Hhex immunostaining at E10.5. (A) In the control, Hhex is detected in the liver bud (outlined in solid line) and extrahepatic biliary tract primordia (outlined in dashed line). The arrow marks the EHBD precursor epithelium and the asterisk labels the gall bladder primordium. (B) In the mutant, Hhex is efficiently deleted in the liver bud (outlined in solid line) and in the presumptive EHBD precursor (outlined in dashed lines). The arrow marks Hhex-negative cells in the EHBD precursor epithelium. The arrowhead labels an endothelial cell. (C,D) HNF6 immunostaining at E10.5. (C) HNF6 is normally expressed in the liver bud (lb) and gall bladder primordium (gb). (D) In the mutant, the gall bladder primordium is absent.

(E,F) Hematoxylin and eosin (H+E) staining at E13.5. (E) Control liver. (F) The *Foxa3-Cre;Hhex^{d2,3/-}* mutant liver is hypoplastic compared to the control. (G-J) CK immunostaining at E13.5. (G) In the control, the EHBD (boxed region) is a duct extending ventro-caudal from the liver to the duodenum, and ventral to the main portal vein (pv). (H) In mutants, the EHBD could not be identified, and in its place were duct-like structures with abnormal epithelium. (I,J) Higher magnification of the boxed regions in E and F respectively. (I) Single cell layer cuboidal epithelium (arrowhead) lines the EHBD (outlined) in the control. (J) In *Foxa3-Cre;Hhex^{d2,3/-}* embryos, abnormal duct-like structures (outlined) were mostly lined by a pseudostratified epithelium (arrowheads), similar to the duodenum (arrow). (K,L) Hhex immunostaining at E13.5. (K) Hhex is expressed in the EHBD epithelium in the control (arrowhead). (L) Hhex is absent from the abnormal duct-like structure, where the EHBD would normally form, in the mutant (arrowhead). Ventral is to the left and all sections are sagittal. mg=midgut, dv=ductous venosus, ht=heart.
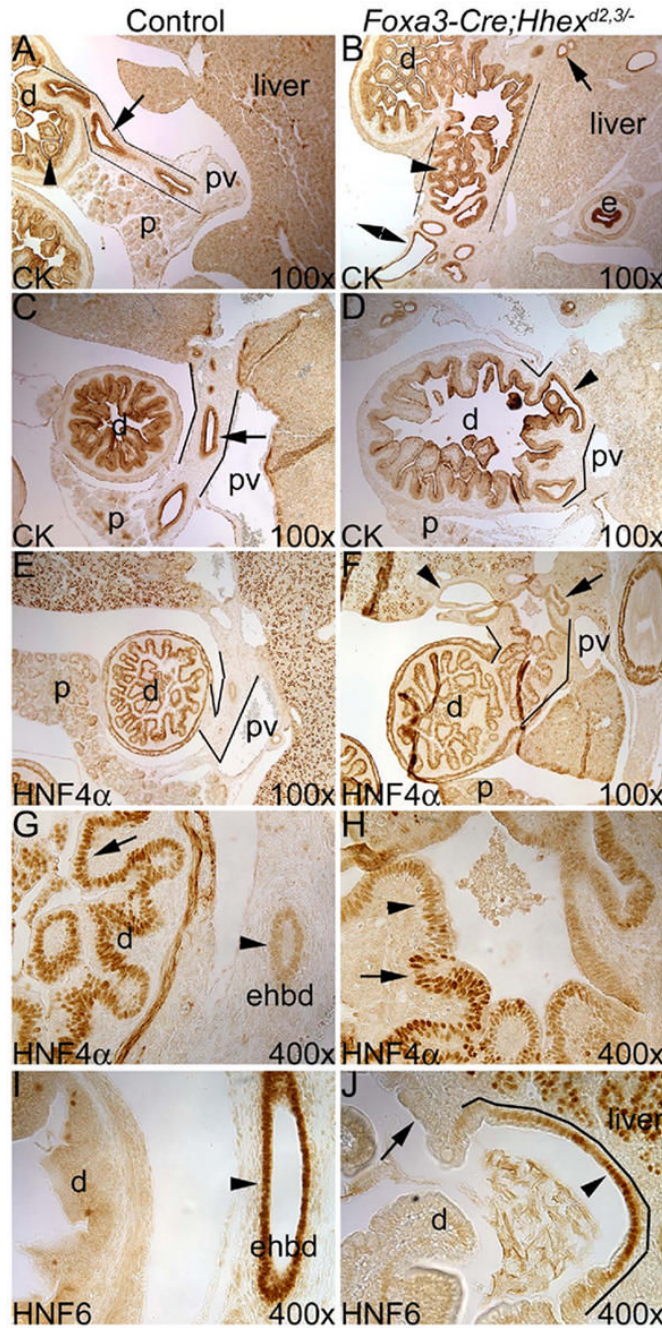
Fig 3. The EHBD was replaced by duodenum in *Foxa3-Cre;Hhex^{d2,3/-}* embryos at E16.5
(A-D) CK immunostaining. (A,B) Transverse sections near the liver hilum. (A) In the control, the arrow marks the cuboidal epithelium of the EHBD (outlined), and the arrowhead shows the large, broad villi of mucosa in the duodenum. (B) In *Foxa3-Cre;Hhex^{d2,3/-}* mutants, duodenal-like epithelium is present at the liver hilum (arrowhead), and directly connects the duodenum to the liver (outlined). Double arrowhead indicates a presumptive dilated hepatic duct, and the arrow marks a biliary cyst within the liver. (C,D) Sagittal section planes near the hilum. (C) In the control, the arrow indicates the single-cell layer epithelium lining the EHBD (outlined). (D) In the mutant, outlines show the presumptive position of the EHBD, which contains mostly pseudostratified columnar epithelial cells with some single cell layer cuboidal

epithelium where the duodenum is connected to the liver (arrowhead). (E-H) HNF4α immunostaining. (E) HNF4α is highly expressed in duodenal epithelium, but is essentially absent in the EHBD (outlined). (F) Abnormal, duodenal-like epithelium (HNF4α-positive) is outlined in the *Foxa3-Cre;Hhex^{d2,3/-}* embryo, and it extends from the duodenum up to the liver hilum. Arrowhead shows a dilated biliary duct within the liver. Arrow marks an abnormal duct within the liver with a pseudostratified epithelium that is positive for HNF4α. (G) In the control, strong nuclear HNF4α expression is detected in duodenal epithelium (arrow), and very weak expression is detected in the EHBD (arrowhead). (H) In the *Hhex* conditional null embryo, strong nuclear expression of HNF4α is detected up to the hilum (arrow), and the arrowhead shows weaker expression in abnormal epithelium within the liver. (I) HNF6 is normally expressed in the EHBD epithelium, but not in the duodenum. (J) In mutants, HNF6 is absent in the ectopic duodenal tissue (arrow), but is detected in some epithelial cells at the base of the liver (arrowhead). These cells probably represent hepatic duct epithelia or a remnant of the EHBD. Ventral is to the left. e=esophagus.
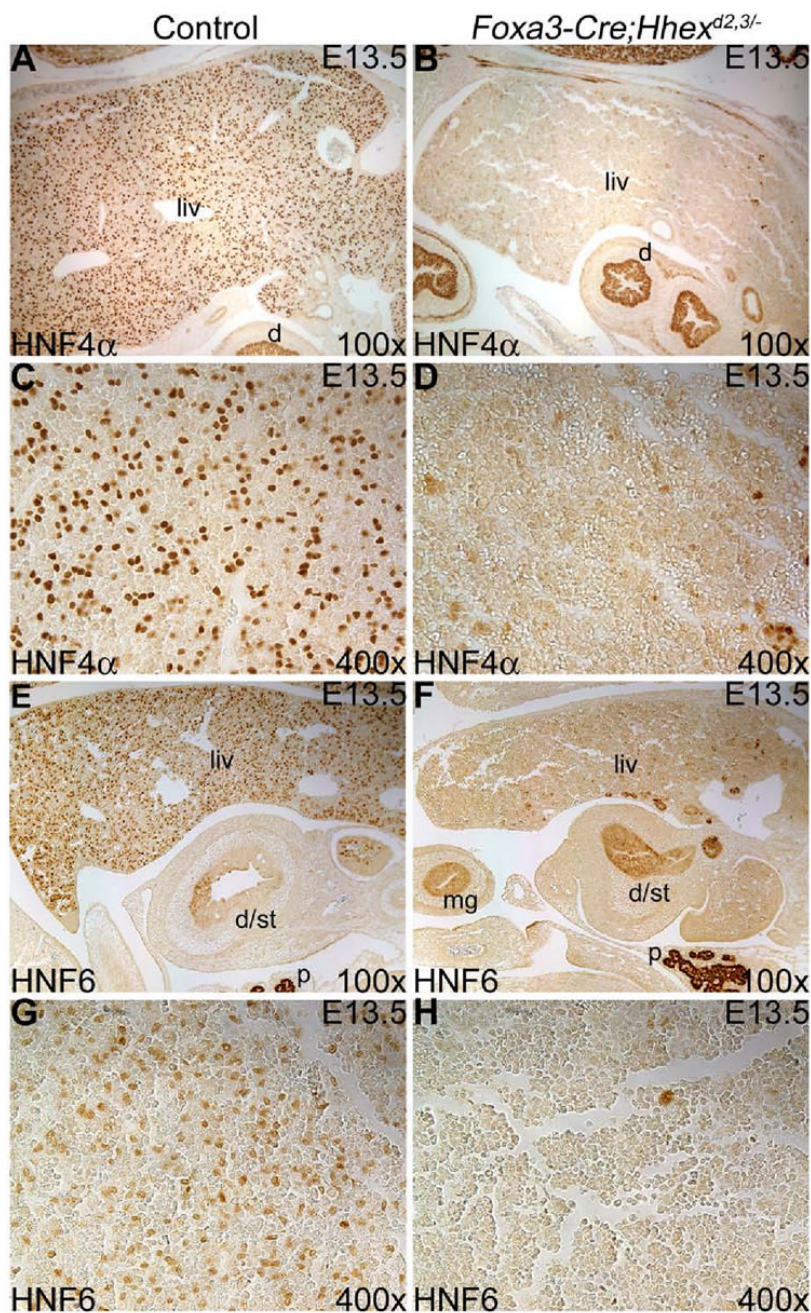
Fig 4. Deletion of *Hhex* in the hepatic diverticulum resulted in an early loss of HNF4α and HNF6 expression in the *Foxa3-Cre;Hhex$^{d2,3/-}$* mutant liver

(A-D) HNF4α immunostaining at E13.5. (A) HNF4α expression in hepatoblasts of the control liver (liv). (B) HNF4α is absent in cells of the *Foxa3-Cre;Hhex$^{d2,3/-}$* mutant liver. (C,D) Higher magnifications of control and mutant livers, respectively. (E-H) HNF6 immunostaining at E13.5. (E) HNF6 is expressed in hepatoblasts of the control liver. (F) In the *Foxa3-Cre;Hhex$^{d2,3/-}$* mutant, HNF6 is absent in almost all hepatic cells. d/st=rostral duodenum/caudal stomach. (G,H) Higher magnifications of control and mutant livers, respectively. Ventral is to the left in A, B, E and F.
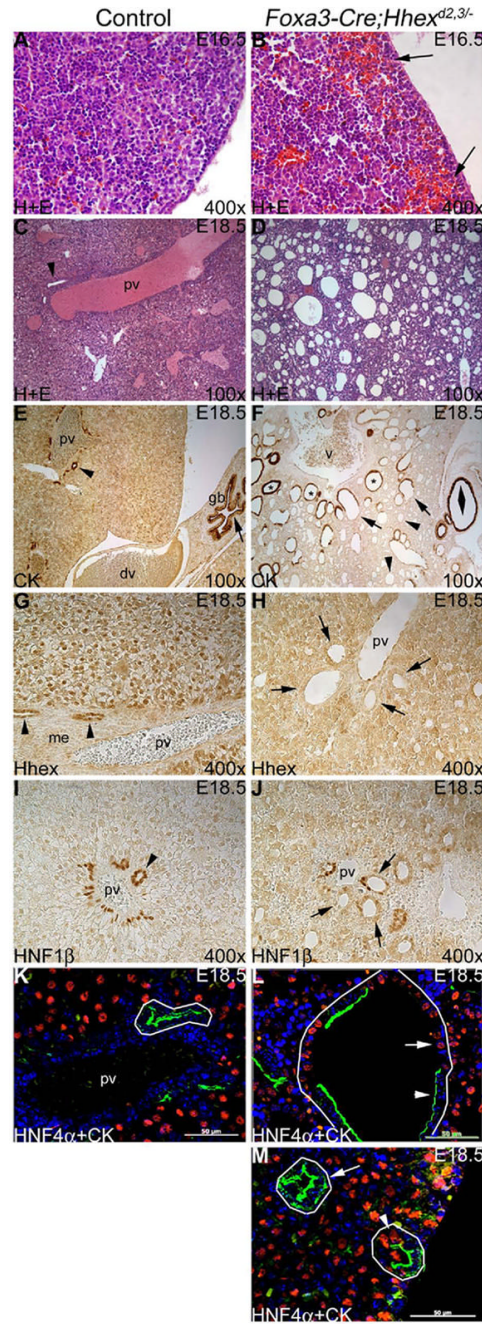
Fig 5. The *Foxa3-Cre;Hhex^{d2,3/-}* mutant liver is severely abnormal by late embryogenesis
(A-D) Hematoxylin and eosin (H+E) staining at E16.5 (A,B) and E18.5 (C,D). (A) Control ventral liver periphery. (B) *Foxa3-Cre;Hhex^{d2,3/-}* mutant ventral liver periphery showing loss of cohesive structure and trapping of blood cells (arrows). (C) A large branch of the portal vein is shown with an IHBD (arrowhead) in the control liver. (D) Severe *Foxa3-Cre;Hhex^{d2,3/-}* mutant liver phenotype. The mutant liver was severely cystic, and hepatic architecture appeared grossly abnormal. (E,F) CK immunostaining at E18.5. (E) Biliary epithelia of the IHBD (arrowhead) and the gall bladder (arrow) are strongly positive for CK. (F) Severe *Foxa3-Cre;Hhex^{d2,3/-}* mutant liver phenotype. Mutant livers had duct-like structures/cysts lined by CK-positive cells (asterisks), CK-negative cells (arrowheads), and both CK-positive and CK-

negative cells (arrows). The large biliary cyst on the right (double arrowhead) is probably derived from cystic duct epithelium. V=presumptive junction of inferior vena cava and main portal vein. Ventral is to the right in E and F. (G,H) Hhex immunostaining at E18.5. (G) Hhex is expressed in biliary cells of IHBD (arrowheads) and in hepatocytes in controls. me=periportal mesenchyme. (H) Hhex is absent in cystic biliary epithelia (arrows) and in hepatocytes in the mutant. (I,J) HNF1β immunostaining at E18.5. (I) In the control, HNF1β is expressed in biliary cells of intrahepatic bile ducts (arrowhead). (J) In the mutant, HNF1β is absent or weakly expressed in most cystic cells (arrows). (K-M) HNF4α (red) + CK (green) dual immunofluorescence at E18.5. (K) In the control, IHBD cells (outlined) are CK-positive and HNF4α-negative. (L,M) *Foxa3-Cre;Hhex$^{d2,3/-}$* mutants. (L) Severe mutant phenotype. A large duct-like structure/cyst (outlined) containing both CK-positive cells (arrowhead) and HNF4α-positive cells (arrow). (M) Biliary cysts (arrow) are detected in the absence of the portal vein, and some hybrid cells (both HNF4α-positive and CK-positive) were also detected in cysts (arrowhead). Hepatocytes (HNF4α-positive) remain small and tightly clustered (compare to control in K). Scale bar=50um.
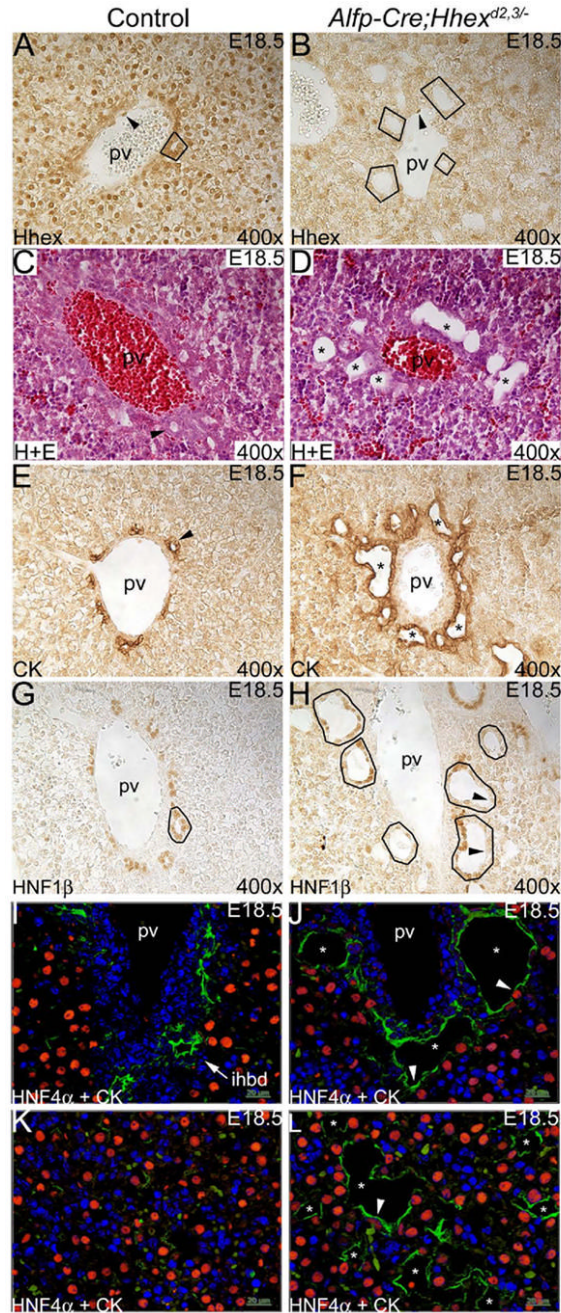
Fig 6. Hhex is required for the proper development of intrahepatic bile ducts
(A,B) Hhex immunostaining on liver sections at E18.5. (A) In controls, Hhex was detected in
hepatocytes, biliary epithelial cells of the IHBD (outlined), and endothelial cells (arrowhead).
(B) In *Alfp-Cre;Hhex^d2,3/-* mutants, Hhex was not detected in liver parenchyma or BEC
(outlined), but Hhex was detected in endothelial cells (arrowhead). (C,D) H+E staining. (C)
Control portal vein with IHBD precursor (arrowhead). (D) Mutant portal vein with abnormal
duct-like structures/cysts (asterisks). (E,F) CK immunostaining. (E) In controls, portal veins
were associated with one or two CK-positive IHBD (arrowhead). (F) In mutants, portal veins
were surrounded by many CK-positive cysts and duct-like structures (asterisks). (G,H)
HNF1β immunostaining. (G) In the control, HNF1β was detected in biliary cells of IHBD

(outlined). (H) In *Alfp-Cre;Hhex*$^{d2,3/-}$ mutants, some cysts had many cells that were HNF1β-negative. (I-L) HNF4α and CK dual-label immunofluoresence. (I) In controls, IHBD cells are CK-positive and HNF4α-negative (arrow). (J) In mutants, many cystic CK-positive cells were also HNF4α-positive (arrowheads). (K) In controls, parenchymal tissue is composed mainly of HNF4α-positive hepatocytes. (L) In mutants, cysts (asterisks) with hybrid cells (arrowhead) were present throughout the liver.

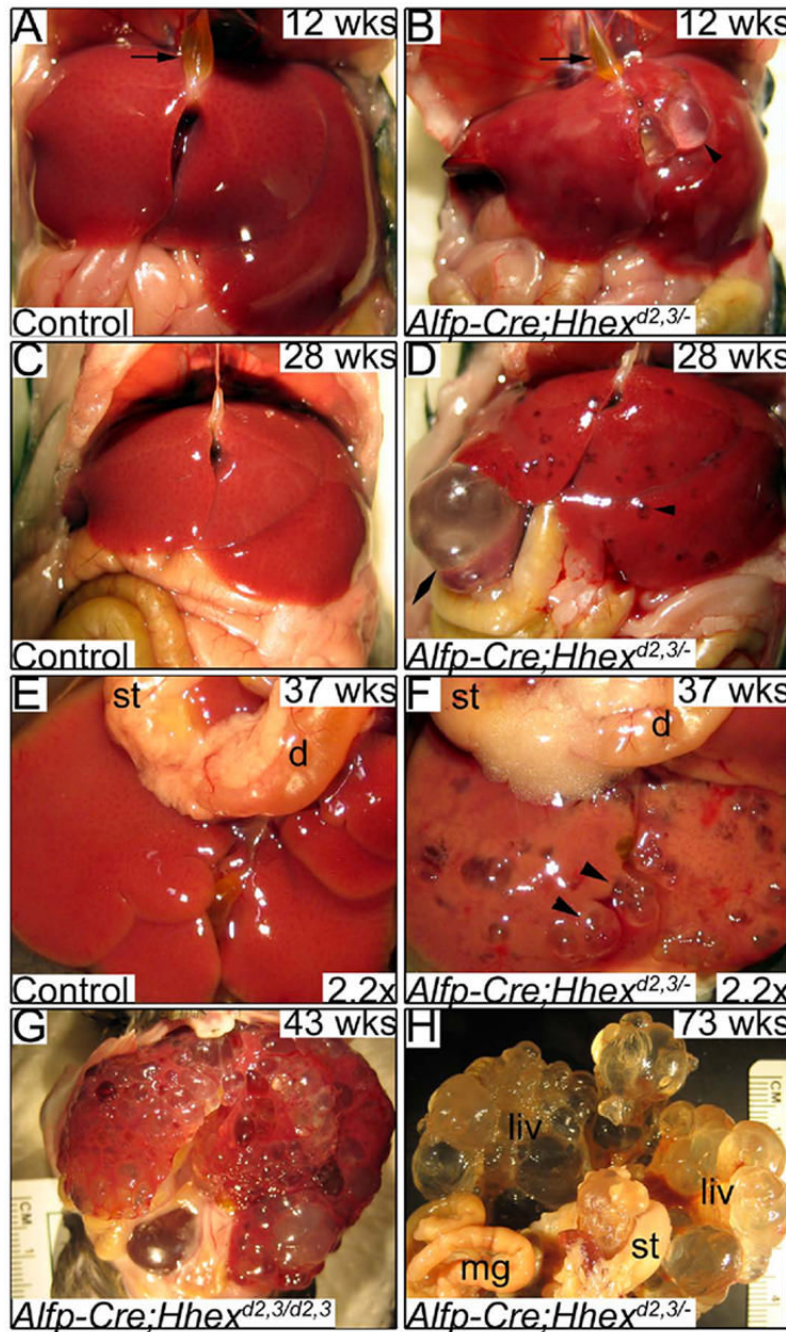**Fig 7. Deletion of *Hhex* in the embryonic liver results in polycystic liver disease in adult mice**
(A,B) Ventral views of livers at 12 weeks of age. (A) Control liver and gall bladder filled with bile (arrow). (B) *Alfp-Cre;Hhex*$^{d2,3/-}$ mutant liver and gall bladder filled with bile (arrow). Arrowhead indicates a large hepatic cyst filled with a clear fluid. (C,D) Ventral views of livers at 28 weeks of age. (C) Control liver. (D) Numerous cysts are obvious throughout the liver (arrowhead). Double arrowhead shows an extremely large hepatic cyst. (E,F) Ventro-caudal views of dissected livers at 37 weeks of age. (E) Control liver. (F) The *Alfp-Cre;Hhex*$^{d2,3/-}$ liver has many large, multi-loculated cysts (arrowheads) and some discoloration, probably due to fibrosis. (G) A mutant liver at 43 weeks of age with severe cystic disease. (F) Ventro-caudal

view of a mutant liver (fixed) at 73 weeks of age, which is almost completely cystic.
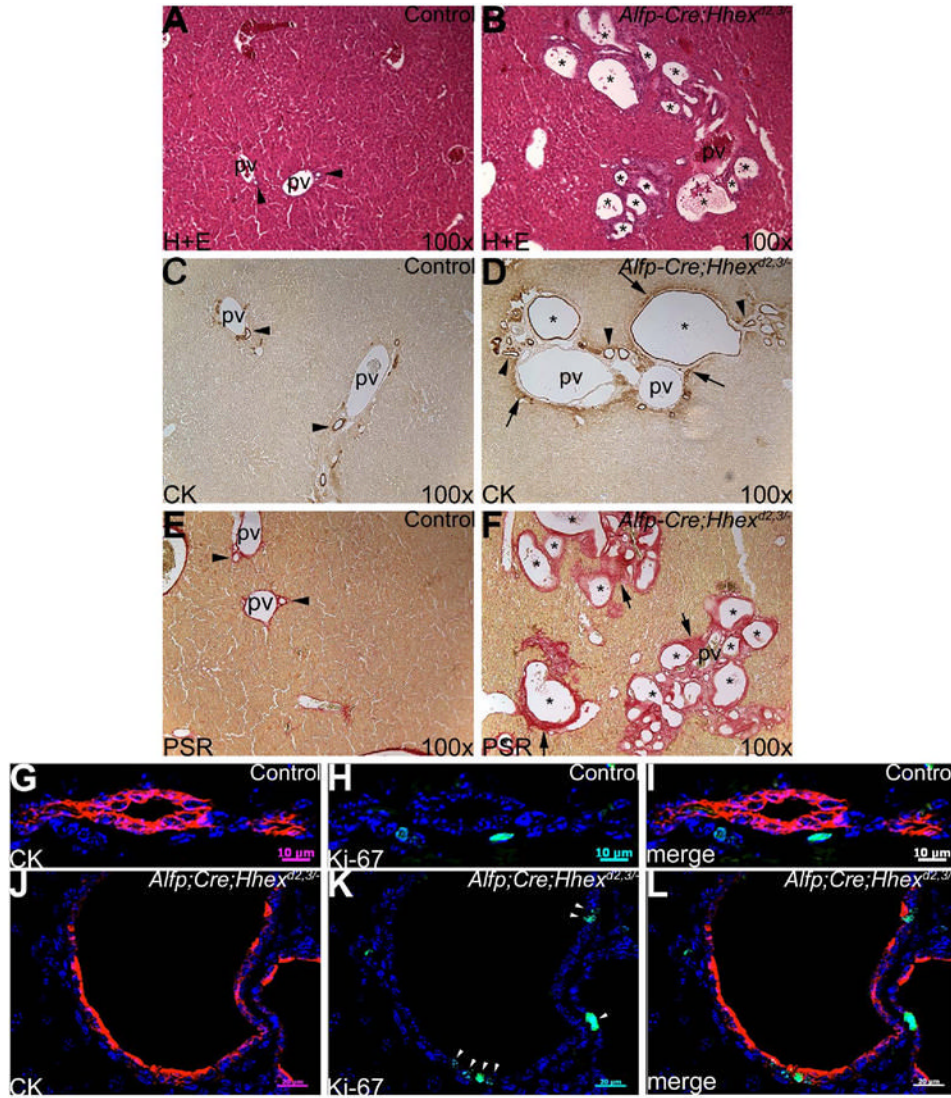st=stomach, d=duodenum.

Fig 8. *Hhex* is required for proper morphogenesis of intrahepatic bile ducts
Histology and immunostaining on liver sections from mice at 28 weeks of age. (A,B) H+E
staining. (A) In controls, portal veins are generally associated with one or two IHBD
(arrowheads). (B) In *Alfp-Cre;Hhex*$^{d2,3./-}$ mutants, portal veins are surrounded by multiple
duct-like structures/cysts (asterisks). (C,D) CK immunohistochemistry. (C) CK expression is
normally confined to biliary epithelial cells lining the IHBD in the periportal region
(arrowheads). (D) Asterisks show large CK-positive biliary cysts surrounding a branch of the
portal vein in the mutant. Arrowheads show multiple smaller ducts/cysts in the periportal
region. Arrows show an up-regulation of biliary-specific CK in periportal cells. (E,F) Picro-
sirius red staining for collagen. (E) In controls, a relatively thin layer of collagen (red staining)
surrounds the IHBD (arrowheads) and portal veins. (F) In mutants, extensive collagen
deposition (arrows) was detected around cysts (asterisks) and portal veins. (G-L) Dual-label
immunofluoresence for CK (red) and Ki-67 (green). (G-I) Control IHBD. (G) CK positive
BEC. (H) In general, BEC are normally Ki-67-negative. (I) Merged image of G and H. Scale
bar is 10um. (J-L) *Alfp-Cre;Hhex*$^{d2,3/-}$ cyst epithelia. (J) Cystic epithelia are CK-positive. (K)
Many cells lining cysts are Ki-67-positive (arrowheads). (L) Merged image of J and K. Scale
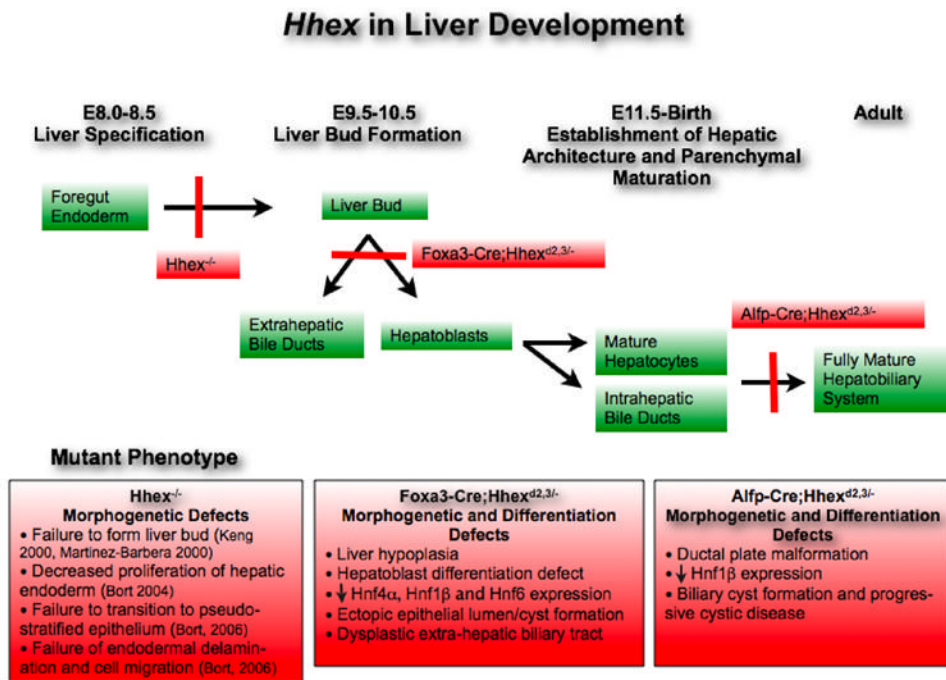bar is 20um.

Figure 9. Summary of the effects of *Hhex* deletion at different stages of liver development
The stages of liver development are noted across the top with the tissues/cell types that express Hhex noted in green boxes. The consequences of *Hhex* deletion as well as the specific *Hhex*-deleted mouse strain are noted and summarized in the red boxes.