sox9b Is a Key Regulator of Pancreaticobiliary Ductal System Development

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Abstract

The pancreaticobiliary ductal system connects the liver and pancreas to the intestine. It is composed of the hepatopancreatic ductal (HPD) system as well as the intrahepatic biliary ducts and the intrapancreatic ducts. Despite its physiological importance, the development of the pancreaticobiliary ductal system remains poorly understood. The SRY-related transcription factor SOX9 is expressed in the mammalian pancreaticobiliary ductal system, but the perinatal lethality of Sox9 heterozygous mice makes loss-of-function analyses challenging. We turned to the zebrafish to assess the role of SOX9 in pancreaticobiliary ductal system development. We first show that zebrafish sox9b recapitulates the expression pattern of mouse Sox9 in the pancreaticobiliary ductal system and use a nonsense allele of sox9b, sox9bfh313, to dissect its function in the morphogenesis of this structure. Strikingly, sox9bfh313 homozygous mutants survive to adulthood and exhibit choledochal association with hepatic and pancreatic duct proliferation, cyst formation, and fibrosis. Analysis of sox9bfh313 mutant embryos and larval reveals that the HPD cells appear to mis-differentiate towards hepatic and/or pancreatic fates, resulting in a dysmorphic structure. The intrahepatic biliary cells are specified but fail to assemble into a functional network. Similarly, intrapancreatic duct formation is severely impaired in sox9bfh313 mutants, while the embryonic endoderm and acinar compartments appear unaffected. The defects in the intrahepatic and intrapancreatic ducts of sox9bfh313 mutants worsen during larval and juvenile stages, prompting the adult phenotype. We further show that Sox9b interacts with Notch signaling to regulate intrahepatic biliary network formation: sox9b expression is positively regulated by Notch signaling, while Sox9b function is required to maintain Notch signaling in the intrahepatic biliary cells. Together, these data reveal key roles for SOX9 in the morphogenesis of the pancreaticobiliary ductal system, and they cast human Sox9 as a candidate gene for pancreaticobiliary duct malformation-related pathologies.

Introduction

The pancreaticobiliary ductal system refers to the complex network of ducts that compose the hepatopancreatic ductal (HPD) system as well as the intrapancreatic and intrahepatic ductal networks. The HPD system consists of the extrahepatic duct, cystic duct, gallbladder, common bile duct, and extrapancreatic duct. It connects to the intrahepatic biliary ducts to enable bile flow and storage. The intrapancreatic ducts collect the digestive enzymes secreted by the pancreatic acinar cells. Pancreatic juice and bile flow to the hepatopancreatic ampulla to be released into the intestine and allow digestion and absorption of nutrients [1]. Malformations of the pancreaticobiliary ductal system impair the function of digestive organs and are associated with various congenital conditions whose causes are mostly unknown.

In mammals, the transcription factor Sox17 is specifically expressed in a segment of the ventral foregut from which the pancreaticobiliary ductal system derives [2]. This factor has been shown to be a master regulator of pancreaticobiliary ductal system formation by specifying, in conjunction with Hhex and Pdx1, different lineages of the liver, pancreas and HPD system [2]. The liver is specified as a group of cells that expresses Hhex but not Sox17 or Pdx1. The intrahepatic biliary network requires several signaling pathways including TGFβ, Notch and Wnt, to differen...
Author Summary

The liver and pancreas function as exocrine glands that secrete bile and pancreatic juice, respectively, to aid the digestion and absorption of nutrients. These fluids reach the intestine via the pancreaticobiliary ductal system, a complex network of ducts. Despite its pivotal role, the development of this ductal system is poorly understood.

We have discovered that the zebrafish transcription factor gene sox9b, like its mammalian ortholog, is specifically expressed in the pancreaticobiliary ductal system. The perinatal lethality of Sox9 heterozygous mice makes the analysis of SOX9 function challenging; thus, we turned to the zebrafish to analyze the role of SOX9 in pancreaticobiliary ductal system development. We found that zebrafish sox9b mutants, which survive to adulthood, display defects in the morphogenesis of this ductal network: the intrahepatic and intrapancreatic ducts fail to form a branched network, whereas the ducts connecting the liver and pancreas to the intestine are malformed. These ductal defects affect bile transport and lead to cholestasis in adult mutant fish. At the molecular level, Sox9b interacts with the Notch signaling pathway to regulate the development of the intrahepatic biliary network. Therefore, our work in zebrafish reveals a broad and complex role for SOX9 in pancreaticobiliary ductal system morphogenesis.

Results

sox9b is expressed in the developing pancreaticobiliary ductal system

Intrigued by the recent data revealing Sox9 expression in the ductal trees of the liver and pancreas as well as in the HPD system in mouse [24], we analyzed the expression pattern of sox9b in zebrafish by in situ hybridization. We found that in addition to the head region and pectoral fins, sox9b is specifically expressed in the pancreaticobiliary ductal system (Figure 1A–1D). At 30 hours post fertilization (hpf), sox9b is expressed in a segment of the foregut endoderm (bracket, Figure 1A) that appears to give rise to the liver bud (arrow, Figure 1B) and the HPD primordium (bracket, Figure 1B). At 60 hpf, sox9b expression becomes evident in the intrahepatic ducts (arrow, Figure 1C) and then extends to the extra- and intrahepatic ducts (white arrow, Figure 1D). In contrast to sox9b, sox9a does not appear to be expressed in the pancreaticobiliary ductal system in zebrafish (Figure S1, left panel). These data show that zebrafish sox9b recapitulates the expression in bile duct, gallbladder and hepatopancreatic ampulla [23–25]. Sox9 belongs to the SRY-related box (SOX) gene family that encodes transcription factors containing an HMG box DNA-binding domain. In humans, SOX9 is expressed in the fetal brain, liver, testis and skeletal tissue [26]. Haploinsufficiency of SOX9 is associated with campomelic dysplasia (CD, OMIM #114290), which is characterized by severe skeletal malformations and sex reversal [26,27]. More recently, it has been shown that SOX9 is also expressed in the early human fetal pancreas and analysis of CD individuals have revealed pancreatic defects including islet hypoplasia and reduction of hormone expression [28]. Consistent with the defects observed in CD patients, heterozygous knock-out mice are perinatal lethal due to skeletal abnormalities [29]. Conditional knock-out mice have been generated to study SOX9 function: pancreas-specific inactivation of Sox9 using Pdx1-Cre reveals a critical role in the maintenance of the pancreatic progenitor pool [25], whereas liver-specific inactivation of Sox9 using Albumin/z-fetoprotein (Alfp)/Cre shows that it is required for the timely maturation of asymmetrical structures to symmetrical biliary ducts [23]. A potential role for SOX9 in HPD development has not yet been investigated due to the lack of a HPD-specific Cre line.

The zebrafish genome contains two sox9 orthologs, sox9a and sox9b, which exhibit partially overlapping expression patterns in the craniofacial cartilage, otic placodes and pectoral appendages [30]. Null mutants of sox9a exhibit cartilage defects that mimic those observed in human CD [31]. Although a similar phenotype has been reported for the sox9b booz mutant [30], the chromosomal deletion which underlies the booz1 lesion removes eleven other genes, greatly limiting the use of this allele to study the function of Sox9b.

Here, we dissect the requirement for Sox9b in the development of the pancreaticobiliary ductal system in zebrafish. We show that similar to mammalian Sox9, zebrafish sox9b is expressed in the pancreaticobiliary ductal system. Detailed phenotypic analysis of a sox9b TILLING mutant reveals that Sox9b regulates the formation of the HPD system as well as the morphogenesis of the intrapancreatic and intrahepatic ducts. Strikingly, the pancreaticobiliary phenotypes observed in larvae worsen during juvenile stages and lead to cholestasis in the homozygous mutant adult fish. We also observed a positive feedback loop between Sox9b and Notch signaling in the developing intrahepatic biliary cells: Notch signaling regulates sox9b expression, and in turn Sox9b is required to maintain Notch activity in the intrahepatic biliary cells.
Figure 1. sox9b is expressed in the pancreaticobiliary ductal system, and the sox9b<sup>fh313</sup> lesion is a nonsense mutation. (A–D) sox9b expression is observed in the head and fin buds (arrowheads in A–C), as well as the notochord (n) and part of the foregut endoderm (bracket) at 30 hpf (A). At 48 hpf, sox9b expression is observed in the liver bud (arrow) and hepatopancreatic duct primordium (bracket) (B). At 60 hpf, sox9b expression covers the intra- (arrow) and extrahepatic (bracket) ducts (C), and extends to the intrapancreatic ducts by 72 hpf (white arrow, D). Dorsal
pattern of mammalian Sox9 in the intrapancreatic and intrahepatic ducts as well as in the HPD system [23–25].

sox9b<sup>fh313</sup> lesion is a nonsense mutation

To investigate the potential role of Sox9b in the formation of the pancreaticobiliary ductal system, we isolated a novel mutation in sox9b<sup>fh313</sup> in collaboration with the Zebrafish TILLING Consortium. Contrary to sox9b<sup>fh313</sup> which consists of a deletion of the lower tip of linkage group 3 [30], sox9b<sup>fh313</sup> is a point mutation located in the first exon of sox9b (Figure 1E). The A to T transition at position 302 leads to a premature stop codon at amino acid Lys68. This nonsense mutation likely leads to the synthesis of a truncated protein that lacks the HMG-box DNA-binding domain. IPD, intrapancreatic duct; IHD, intrahepatic duct; HPD, hepatopancreatic duct; HMG, high-mobility group; aa, amino acid.

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sox9b mutants survive to adulthood and exhibit cholestasis associated with hepatic and pancreatic fibrosis and duct dilation

In contrast to a previous report describing the phenotypes of sox9b<sup>fh313</sup> mutants and sox9b morpholino-injected embryos [30], homozygous sox9b<sup>fh313</sup> mutants exhibit a normal external morphology and do not show a curly-down body axis or craniofacial defects (data not shown). sox9b mutants survive to the adult stage but are much thinner than their wild-type or heterozygous siblings (data not shown). Dissection of the digestive system of 5-month-old sox9b mutants revealed preserved anatomical relationships, including a three-lobed liver and correctly-looped intestine; however, both the liver and pancreas were strikingly dark green suggesting abnormal bile accumulation (cholestasis) in these organs (Figure 2A–2B). Hematoxylin-and-eosin staining of histological sections of mutant organs showed that both organs exhibited lesions with extensive proliferation and dilation of the ducts, which were surrounded by fibrotic tissue (Figure 2C–2D). Interestingly, in the liver, ductal defects were restricted to the region that connects to the extrahepatic ductal system (dashed rectangle, Figure 2D) whereas the rest of the organ was much less affected. In contrast, ductal defects in the pancreas were present throughout the organ and worsened towards its distal part (Figure 2D). In the pancreas, the acinar compartment was greatly reduced and secondary islets could not be detected in the sections examined (Figure 2D).

Sox9b is required for the patterning and differentiation of the HPD system

Due to the robust and highly conserved expression of sox9b in the pancreaticobiliary ductal system and the striking liver and pancreas phenotypes seen in the adult mutant fish, we decided to further investigate the roles of Sox9b in the development of these tissues. In zebrafish, the HPD system exhibits unique gene expression profiles that separate it from the liver and pancreas starting at early developmental stages [15]. At 50 hpf, the primordium of the HPD system can be distinguished by strong labeling with the 2F11 antibody, whose antigen remains to be identified [15,32] (bracket, Figure 3A, 3A′), and low expression of the transcription factor Prox1 [15] (bracket, Figure 3C). In contrast, the liver and pancreas exhibit moderate labeling of 2F11 (Figure 3A, 3A′, but high expression of Prox1 (Figure 3C). In sox9b mutants, 2F11 labeling was mostly absent from the region where the presumptive HPD primordium resides (bracket, Figure 3B, 3B′). Moreover, we observed elevated expression of Prox1 in the same region (bracket, Figure 3D). 2F11 labeling showed that by 80 hpf, the HPD system in wild-type larvae has developed into different compartments, including the extrahepatic duct, cystic duct, common bile duct, and gallbladder [15] (Figure 3E, 3E′). At the equivalent stage, the differentiation of the HPD system had partially recovered in sox9b mutants as suggested by 2F11 labeling. However, it was severely dysmorphic, with no clear morphological distinction between the cystic duct, extrahepatic duct, and common bile duct (Figure 3F, 3F′). Furthermore, the mutant HPD system often seemed to intrude into the liver (Figure 3F), which was never observed in wild-type larvae. These data indicate that the HPD primordium in sox9b mutants exhibits patterning and differentiation defects.

Concordant with the dysmorphic HPD system, the gallbladder in sox9b mutants was often indistinguishable based on morphology (Figure 3F and Figure S3A, S3B). We analyzed the expression of sox17 which marks the gallbladder and its primordium from 36 hpf to 5 days post-fertilization (dpf) [33], and found that it was greatly reduced or absent in sox9b mutants at 52 hpf (Figure 3G) and that it did not recover during later development (Figure 3H). This defect in sox17 expression supports the notion that gallbladder development is severely impaired in sox9b mutants.

sox9b mutants fail to form a complex network of ducts in the pancreas

We then addressed the role of Sox9b in intrapancreatic duct formation by using the double transgenic line Tg(Tp1bglob:GFP) ; Tg(Tp1bglob:H2B-mCherry) that expresses both GFP and H2B-mCherry under the control of a Notch-responsive element [34,35]. This line allows the visualization of the shape and nuclei of the intrapancreatic duct cells, as indicated by the overlapping expression of these fluorescent proteins with ductal markers such as E-cadherin and 2F11 [34,35]. Intrapancreatic ducts derive from cells within the ventral pancreatic bud that migrate towards, and eventually surround, the principal islet at 48 hpf [22]. From 60 hpf, ductal progenitors start to migrate caudally to form a row of cells that give rise to the main intrapancreatic duct [22] (Figure 4A–4A′). The migration of the ductal progenitors did not seem to be impaired in sox9b mutants; however, the number of cells within the intrapancreatic ducts was significantly reduced (Figure 4B–4B′, 4G). In wild-type larvae, at 100 hpf, the pancreatic tail keeps elongating, the number of ductal cells has slightly increased (Figure 4C–4C′, 4G) and secondary branches (arrowheads, Figure 4C–4C′) start to form from the...
main duct. In contrast, in sox9b mutants, the number of ductal cells did not increase from 80 to 100 hpf, and no secondary branches appeared, resulting in a primitive ductal system (Figure 4D–4D'). At 120 hpf, the ductal network in wild-type larvae has become more complex with numerous secondary branches (arrowheads, Figure 4E') spreading over the acinar compartment (Figure 4E–4E'). In contrast, the intrapancreatic ductal system in sox9b mutants remained poorly developed and clusters of cells could be observed along the main duct (Figure 4F–4F'), which was still devoid of secondary branches. These data indicate that fewer intrapancreatic duct cells differentiate in the mutants and those that do fail to undergo branching morphogenesis. Furthermore, the number of ductal cells in sox9b mutants did not increase as in wild-types. Such a defect is likely due to a problem with cell differentiation as we did not observe any obvious differences in ductal cell proliferation or survival between wild-types and sox9b mutants (data not shown).

Given that the adult mutant pancreas exhibits a loss of acinar and potentially endocrine tissues, we analyzed the formation of these compartments during larval and juvenile stages. As assessed by Elastase staining, sox9b mutants showed apparently normal proportion of pancreatic acinar tissue at all stages analyzed (Figure 4B, 4D, 4F and Figure S2B', S2D', S2F'). For the endocrine tissue, we investigated the morphology of the primary islet by analyzing TgBAC(neurod;GFP) expression which marks early endocrine cells [36]. At late larval (7 dpf) as well as juvenile (2 and 3 weeks) stages, the area of the primary islet appeared similar in wild-type and sox9b mutant animals (Figure 4H), suggesting that Sox9b is not required for primary islet formation. At 4 weeks of age, the sox9b mutant primary islet was half the size of the wild-type primary islet (Figure 4H). However, it is important to note that at this stage, sox9b mutant pancreata were also much less developed than wild-type pancreata (Figure S2E', S2F'). Indeed, sox9b juvenile mutants often exhibit growth retardation compared to wild-types and thus, the smaller size of the primary islet could be attributed to an overall growth defect.

In addition to the primary islet, we investigated the formation of secondary islets that arise from progenitors in the intrapancreatic ducts [34,35] and that, during larval and juvenile stages, appear as small clusters of delaminated cells [34]. We decided to also count single TgBAC(neurod;GFP)-positive cells that recently delaminated from the ducts and assumed an endocrine fate. Hence, counting the number of TgBAC(neurod;GFP)-positive cell/cluster number in sox9b mutants (Figure 4I). Given that the mutant intrapancreatic ductal network remained primitive and failed to expand at juvenile stages (Figure S2D', S2F'), the defect in secondary islet formation could be related to the lower number of progenitors within the pancreas.

Figure 2. Adult sox9b mutants develop cholestasis associated with fibrosis, duct proliferation, and dilation in both the liver and pancreas. (A–B) Dissection of the digestive system of 5 month-old wild-type (A) and sox9b homozygous mutant (B) fish reveals a green mutant liver and pancreas reflecting the accumulation of bile in both organs. Anterior to the top. (C–D) Hematoxylin-and-eosin (H&E) staining of histological cross-sections of wild-type (C) and sox9b mutant (D) digestive tracts shows abnormal duct morphology in the mutant liver and pancreas. These duct malformations are focalized around the connection with the extrahepatic duct (dashed rectangle in D) in the liver, whereas they are spread over the entire pancreas. Higher magnifications of liver (C–D') and pancreas (C–D') reveal dilated ducts surrounded by fibrotic tissue (pink staining in D' labeled as "f") in both organs in sox9b mutants. Li, liver; Pa, pancreas; I, intestine; Pi, primary islet; a, acinar compartment. Scale bars, 1 mm in A–B; 500 μm in C–D; 50 μm in C–D'.
Altogether, these data indicate that Sox9b function is required for the development of the intrapancreatic ductal system as well as directly or indirectly for the formation of secondary islets.

Sox9b regulates intrahepatic biliary duct morphogenesis and bile canaliculi formation

To examine the role of Sox9b in intrahepatic biliary development, we used the Tg(Tp1bglob:GFP) line which also marks the intrahepatic biliary cells [21,35]. During zebrafish liver development, the intrahepatic biliary cells undergo significant morphological changes, whereby these initially contiguous cells separate from one another and interconnect via cytoplasmic processes [21]. By 96 hpf, the wild-type intrahepatic biliary system is composed of a lattice-like network of long ducts joined by short interconnecting ducts [21] (Figure 5A). At the equivalent stage, sox9b mutant livers contained similar numbers of intrahepatic biliary cells and hepatocytes as wild-type (data not shown), suggesting that differentiation of the biliary cells is not affected in the mutants. We did not detect any apoptosis of the biliary cells in wild-type or mutant larvae. Strikingly, we observed that most of the biliary cells in the mutant livers failed to separate from one another (Figure 5B).

We quantified the percentage of single intrahepatic biliary cells versus cells in clusters of two, three or four and more cells, and found a significant decrease in the percentage of single intrahepatic biliary cells in sox9b mutants compared to wild-types concomitant with a significant increase in the percentage of cells in clusters of four and more cells (Figure 5C). Moreover, the long bile ducts in the mutants appeared to be wider than those in wild-types (diameters of the mutant ducts: 3.5 \( \mu m \) or wider; wild-type ducts: 2.5 \( \mu m \) or thinner), and were less branched (Figure 5D).

We then used the Tg(fabp10:ras-GFP) line [37] to analyze hepatocyte organization, and co-labeled the animals with an antibody against the bile transporter BSEP to mark the bile canaliculi [38] (Figure 5E, 5G). At 96 hpf, hepatocytes in wild-type livers are arranged as tubules surrounding intrahepatic biliary ducts [20] (Figure 5E). Bile canaliculi are located on the hepatocyte apical membrane which can be marked by the activated leukocyte cell adhesion molecule Alcam [39]. However, in sox9b mutants, hepatocytes often formed spherical rosettes with bile canaliculi and Alcam expression located in the center.

**Figure 3. sox9b mutants display defective HPD patterning during organ development.** (A–B) Labeling for the hepatocyte marker Tg(fabp10:ras-GFP) (green) and the HPD marker 2F11 (red) in wild-type and sox9b mutant embryos at 50 hpf. 2F11 labeling in the HPD primordium is noticeably reduced in sox9b mutants. (A’–B’) Same views as (A–B), but only showing the 2F11 immunostaining. (C–D) In wild-type, Prox1 expression marks the liver and pancreas and is largely absent from the HPD primordium (C). In contrast, Prox1 is abnormally expressed in the HPD primordium in sox9b mutants (D). (A’–B’, C–D) Brackets mark the HPD primordium. (E) By 80 hpf, different compartments of the HPD system, including the cystic duct (CD), common bile duct (CBD), gallbladder (GB), extrahepatic duct (EHD) have become evident in wild-type. (F) In sox9b mutants, the HPD system is dysmorphic and its compartments are indistinguishable based on morphology. The gallbladder is also often missing. (E’–F’) Same views as (E–F), but only showing 2F11 immunostaining. (G–H) Whole-mount in situ hybridization showing sox17 expression in the gallbladder primordium in wild-type and sox9b mutants at 52 (G) and 72 (H) hpf. sox17 expression is greatly reduced or absent in sox9b mutants. The proportion of mutants showing the corresponding phenotype is indicated. Arrows point to the gallbladder primordium. (A–F, A’–F’) All images are projections of confocal z-stacks. Ventral views. (G–H) Dorsal views. Anterior (A) to the top. Pa, pancreas; Li, liver; EPD, extrapancreatic duct. Scale bars, 20 \( \mu m \).

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**Figure 4. sox9b mutant larvae fail to form a complex intrapancreatic ductal network.** (A–F) Confocal images of Tg(Tp1bglob:GFP);Tg(Tp1b-glob:H2B-mCherry) wild-type (top row) and sox9b mutant (bottom row) pancreata at 80 (A,B), 100 (C,D) and 120 (E,F) hpf. Elastase antibody staining (blue) labels acinar cells. Although acinar and endocrine tissues appear morphologically unaffected in sox9b mutants (data not shown), the intrapancreatic ductal network is less complex and secondary branches are missing in the mutants (D–D) whereas they start to form by 100 hpf in wild-type larvae (arrowheads and insets, C–C). (E–F) Higher magnifications of the area marked by dashed squares in (E–F) show that at 120 hpf the main duct forms secondary branches (arrowheads) in wild-type larvae (E–E′), whereas in the mutants, secondary branches remain absent and...
clusters of ductal cells are sometimes observed (F’–F”). (A–F) All images are projections of confocal z-stacks. Ventral views, anterior (A) to the top. Scale bars, 50 μm. (G) Graph representing the number of Tg(Tp1bglob:GFP);Tg(Tp1bglob:H2B-mCherry)-double positive cells (average ± SEM) in the intrapancreatic ducts of wild-type and sox9b mutant larvae at different time points. 7 to 11 larvae of each genotype were counted at each stage. (H) Graph representing the area (in arbitrary unit, a.u.) of the primary islet (average ± SEM) of TgBAC(neurod:GFP)-wild type and sox9b mutant larvae (7 dpf) and juvenile animals (2, 3 and 4 weeks (wks)). 6 to 11 animals of each genotype were analyzed at each stage. Area of primary islet was determined using ImageJ. (I) Graph representing the number of TgBAC(neurod:GFP)-positive cells/ clusters (average ± SEM) along the intrapancreatic ducts (IPD) in wild-type and sox9b mutant larvae (10 dpf) and juvenile animals. 7 to 11 animals of each genotype were analyzed at each stage. Asterisks indicate statistical significance: *p < 0.05; **p < 0.01; ***p < 0.0005; ****p < 0.0001; *****p < 0.00005.
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Loss of Sox9b function causes severe defects in bile transport

To determine whether the cholestasis-like phenotype observed in adult sox9b mutants occurred during early larval development, we administered fluorescent lipid analogs used to visualize bile transport [40] to 6 dpf-old wild-type and mutant larvae. These fluorescent analogs consist of fatty acids with acyl chains of 5- and 2-carbons (C5:0 and C2:0, respectively) tagged with the BODIPY fluorophore. We selected these two analogs because of the different cells and subcellular details each analog reveals following ingestion. BODIPY-FL C5:0 reveals a high degree of subcellular detail in hepatocytes and acinar cells, such as lipid droplets and zymogen granules, as well as in the ductal networks in the liver and pancreas. The shorter BODIPY-FL C2:0 illuminates the hepatic and pancreatic ducts, as well as the gallbladder providing a functional readout of gallbladder and ductal integrity.

Wild-type larvae fed BODIPY-FL C2:0 exhibited a strong fluorescence signal in their gallbladders (Figure S3A), indicating that bile production, drainage and accumulation was normal. Conversely, no gallbladder BODIPY-FL C2:0 signal was observed in sox9b mutants, consistent with their defective gallbladder development (Figure S3B). In the pancreas, BODIPY-FL C2:0 fluorescence was detected throughout the entire intrapancreatic ductal network in wild-type larvae (Figure S3E, S3E’), while it was restricted to the anterior region of the pancreas in sox9b mutants (Figure S3F, S3F’), suggesting that the distal intrapancreatic ducts were not functional. Moreover, we observed large pools of fluorescent fluid accumulating in the mutants’ pancreatic tail (Figure S3F), which is not typically observed in wild-type larvae unless the gallbladder ruptures. This abnormal extracellular accumulation of fluid (likely pancreatic juice or bile) in and around sox9b mutant pancreata is consistent with their malformed pancreaticobiliary ductal system.

Administering BODIPY-FL C5:0 to sox9b mutant livers confirmed the dilation and lack of branching morphogenesis of the intrapancreatic biliary ducts described above as well as the deformation of bile canaliculi in hepatocytes (Figure S3C, S3C’, S3D, S3D’). Taken together, these data support the notion that the cholestasis-like phenotype observed in the adult sox9b mutants results from defects in early ductal morphogenesis.

Notch regulates sox9b expression during intrahepatic biliary network formation

The defects in intrahepatic biliary ducts and bile canaliculi observed in sox9b mutants are strikingly similar to those reported in the mouse and zebrafish models of Notch deficiency [6,9,20,21,41]. In particular, it has been shown in zebrafish that Notch signaling directs the segregation of intrahepatic biliary cells between 70 and 96 hpf [21]. Given that the intrahepatic biliary cells in sox9b mutant livers fail to separate from one another, we hypothesized that Sox9b interacts with Notch signaling to regulate the morphogenesis of the intrahepatic biliary ducts. To test whether inhibiting Notch signaling affects sox9b expression, we treated wild-type and sox9b heterozygous animals with a low dose of the γ-secretase inhibitor DAPT from 75 to 99 hpf [42], and assessed sox9b expression by in situ hybridization (Figure 6A–6E). Such DAPT treatment caused a reduction in sox9b expression which was more pronounced in sox9b heterozygotes than in wild-types. We also performed the reverse experiment by using Tg(hsp70l:Gal4);Tg(UAS:myc-Notch1a-intra) hemizygous larvae to induce ubiquitous overexpression of the Notch intracellular domain (NICD) upon heat-shock treatment [43]. These animals were heat-shocked at 80 hpf and sox9b expression was analyzed 26 hours later by in situ hybridization. The heat-shock treatment efficiently induced overactivation of Notch signaling activity in the Tg(Tp1bglob:GFP); Tg(Tp1bglob:H2B-mCherry) larvae (Figure 6H, 6I). We observed an increase in sox9b expression throughout the pancreas, liver and HPD system (bracket) in the double-transgenic larvae compared to their single-transgenic control siblings (Figure 6F, 6G). Expression of sox9b could even be detected in the gallbladder (arrowhead, Figure 6G), which was not seen in control larvae (Figure 6F). This increase in sox9b expression is unlikely due to an NICD-induced proliferation of sox9b-positive cells because we could already detect higher levels of sox9b expression as early as four hours after heat-shock treatment (data not shown). Taken together, these loss- and gain-of-function analyses reveal that Notch signaling regulates sox9b expression during intraperitoneal biliary duct morphogenesis. These data are consistent with studies in mouse showing that Notch1 can directly inhibit the Sox9 promoter [9].

Sox9b is required to maintain Notch signaling in the intrahepatic biliary duct cells

To further analyze the dynamics of Notch signaling in sox9b mutants, we utilized Tg(Tp1bglob:H2B-mCherry);Tg(Tp1bglob:Venus-Pest) animals in which the Notch-responsive element drives the expression of both H2B-mCherry and VenusPest fluorescent proteins. Contrary to H2B-mCherry which is very stable, the destabilized fluorescent protein VenusPest has a short half-life (2 hours for GFP-Pest in mammalian cells) [44]. Thus, the
sox9b in Pancreaticobiliary Duct Development

Figure 5. Intrahepatic biliary network morphogenesis and bile canaliculi formation are impaired in sox9b mutants. (A–B) Distribution of hepatocytes and biliary cells in 96 hpf wild-type and sox9b mutant larvae as revealed by Prox1 (red) and Tg(Tp1bglob:GFP) (green) expression. In wild-type, the cell bodies of biliary cells are separated from one another and interconnected via cellular processes (A). In sox9b mutants, the biliary cells are clustered together (B). Dashed lines mark the long bile ducts and arrows in (A) point to the short interconnecting ducts that are missing in the mutants. (C) Percentage (average ±SEM) of biliary cells that exist as single cells, doubles, triples, or clusters of four or more at 96 hpf. 6 wild-type and 6 sox9b mutant larvae were analyzed. (D) Number (average ±SEM) of branching points observed along the bile ducts per liver. 7 wild-type and 7 mutants were analyzed. (C–D) Asterisks indicate statistical significance: *p<0.05; **p<0.001; ***p<0.0001. (E, G) Wild-type and sox9b mutant livers stained for BSEP (red) which marks the canaliculi, Alcam (blue) which marks the apical side of the hepatocytes, and Tg(fabp10:ras-Grp) (green) which labels the hepatocytes. The hepatocytes in wild-type larvae are organized in parallel arrays (highlighted by pseudo-colors), while the sox9b mutant hepatocytes are arranged in rosettes (highlighted by pseudo-colors). (F, H) High magnification confocal images showing the morphology of biliary cells and bile canaliculi. The canaliculi (arrows) in sox9b mutant livers appear shorter and wider compared to wild-type. (A–B) All images are projections of confocal z-stacks. (E–H) Single confocal plane images. (A–B, E–H) Ventral views, anterior (A) to the top. Scale bars, 20 μm.

To follow up this observation, we treated wild-type and mutant larvae with 50 μM DAPT, a dose that only partially inhibits Notch signaling. Upon treatment between 106 and 154 hpf, DAPT-treated wild-type larvae showed a specific loss of Notch signaling activity in the proximal (p) rather than the distal (d) region of the liver (Figure S4C). In DAPT-treated mutant larvae, we observed a drastic loss of Notch signaling throughout the entire liver (Figure S4D, S4E), indicating that sox9b mutant biliary cells are more likely to lose Notch signaling activity than wild-type cells. Altogether, these data indicate that sox9b is required for the maintenance but not the initiation of Notch signaling in the intrahepatic biliary cells. Considering our previous data showing that Notch signaling regulates sox9b expression, we hypothesize that Notch and Sox9b interact in a positive feedback loop to ensure the development of the intrahepatic biliary network.

To better understand the biological significance of Notch responsiveness during intrahepatic biliary duct morphogenesis, we compared the distribution of Tg(Tp1bglob:H2B-mCherry)-single positive cells and Tg(Tp1bglob:H2B-mCherry);Tg(Tp1bglob:VenusPest)-double positive cells in wild-type larvae. At 123 hpf, 89% of the double-positive cells existed as individual cells connecting to one another through cellular extensions (Figure 7C; 467 cells in 5 larvae were analyzed). On the other hand, 60% of the Tg(Tp1bglob:H2B-mCherry)-single positive cells, which had switched off Notch signaling, were intermingled with each other to form larger groups (Figure 7C, arrows; 110 cells in 5 larvae were analyzed). Interestingly, these clusters of Tg(Tp1bglob:H2B-mCherry)-single positive cells were mostly present in the multicellular large bile ducts contiguous with the extrapancreatic duct, whereas the individual Tg(Tp1bglob:H2B-mCherry);Tg(Tp1bglob:
Figure 6. Notch signaling regulates sox9b expression in the intrahepatic and intrapancreatic ducts. (A–D) Whole-mount in situ hybridization (ISH) showing sox9b expression in larvae obtained from a wild-type to sox9b heterozygote cross that were treated with DMSO control or 20 μM DAPT between 75 and 99 hpf. In DMSO-treated controls, sox9b was strongly expressed in the liver and pancreas at 99 hpf (A, Class I). In animals treated with 20 μM DAPT (B–D), some showed a slight reduction of sox9b expression in the liver (L) and pancreas (Pa) (B, Class II), others only retained expression of sox9b in the liver (C, Class III), and the remaining ones did not exhibit any obvious expression of sox9b in either organ (D, Class IV). Dorsal views, anterior (A) to the top. (E) Percentages of larvae showing different classes of phenotypes. sox9b heterozygotes exhibited a more severe reduction in sox9b expression than wild-type upon DAPT treatment. The numbers of larvae analyzed are indicated at the bottom. (F–I) Larvae obtained from crossing Tg(msp70:Gad4);Tg(UAS:myc-Notch1a-intra) hemizygous and Tg(Tp1:glob:GFP);Tg(Tp1:glob:2H2BmCherry) parents were heat-shocked at 80 hpf to induce myc-Notch1a-intra expression and fixed 26 hours later (myc-Notch1a-intra)-overexpressing larvae were selected based on the increased expression of both Tg(Tp1:glob:GFP) and Tg(Tp1:glob:2H2BmCherry), and their genotype was confirmed by anti-myc antibody labeling (data not shown). (F–G) in situ hybridization showed an increased expression of sox9b in the liver (L) and pancreas (Pa) as well as in the HPD system (bracket) including the gallbladder (arrowhead) in (myc-Notch1a-intra)-overexpressing larvae (G) compared to (myc-Notch1a-intra)-negative siblings (F). 10 larvae of each genotype were analyzed. Dorsal views, anterior (A) to the top. (H–I) Confocal images of (myc-Notch1a-intra)-negative (H) and (myc-Notch1a-intra)-overexpressing (I) larvae showing elevated expression of Tg(Tp1:glob:GFP) and Tg(Tp1:glob:2H2BmCherry) in the liver (L), pancreas (Pa), gut (g) and gallbladder (gb) in (myc-Notch1a-intra)-overexpressing larvae after heat-shock. Dashed lines in (I) mark the pancreas. All images are projections of confocal z-stacks. Ventral views, anterior (A) to the top. Scale bars: 50 μm.

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biliary ductal system formation, an important but understudied process. We showed that loss of Sox9b function leads to mispatterning of the HPD primordium. Fgf10 signaling also plays a pivotal role in the formation of the HPD system in zebrafish [15]. Zebrafish fgf10 is expressed in the mesenchyme surrounding the HPD system and intestine but not that surrounding the liver or pancreas [17]. fgf10 mutants show a dysomorphic HPD with a reduction or loss of the common bile duct as well as reduced extrapancreatic and extrapapillary ducts. Moreover, fgf10 mutants misexpress hepatic markers such as Prox1 and Hnf4 in their HPD system and pancreas, leading to the ectopic differentiation of some cells in these organs towards a liver fate. Based on the similarities of the fgf10 and sox9b mutant phenotypes, it is possible that Fgf10 and Sox9b interact; for example, Fgf10 signaling could induce or maintain sox9b expression in the HPD primordium to modulate the patterning of this tissue. However, we did not observe any obvious change in sox9b expression in the HPD upon Fgf receptor pharmacological inhibition treatment, nor in fgf10 expression in the surrounding mesenchyme in sox9b mutants. Thus, Fgf10 and Sox9b functions might intersect in other ways.

Our data show that Sox9b is involved in gallbladder development, whose primordium specifically expresses sox17. sox17 is expressed in all endodermal cells during gastrulation [43] and starts to be reexpressed at 36 hpf in a small region of the liver close to the extrahepatic duct [33]. It is then detected in the gallbladder at 60 hpf where it persists until 5 dpf. It will be interesting to determine whether Sox9b directly regulates sox17 expression and also to identify the additional factors involved in inducing sox17 expression in a subset of sox9b positive cells.

The intrapancreatic ductal network is severely disrupted in sox9b mutant larvae, leading to the formation of dilated ducts surrounded by fibrotic tissue in mutant adults. At first glance, this adult phenotype is reminiscent of the one caused by pancreas-specific inactivation of mouse Sox9 [25]; however, given that the pancreatic remnants described in the mouse mutants come from unrecombined Sox9+ progenitor cells, the zebrafish adult phenotype appears much milder than the one seen in mouse. The discrepancy between the two models could be explained by differences during early pancreas development. Indeed, mouse SOX9 is expressed in pluripotent pancreatic progenitors and is required to stimulate their proliferation and survival [25]. However, in zebrafish at the earliest stages of pancreas development, endocrine cells derive first from the endodermal epithelium [18] and then from the extrapancreatic duct [46]; only during larval stages, do endocrine cells derive from progenitors within the intrapancreatic ducts [34], likely following mechanisms similar to those regulating the secondary transition in mouse. Analysis of zebrafish sox9b expression at early stages suggests that it is present in a subset of ventral pancreatic bud cells, which may correspond to the ductal progenitors. If this interpretation is correct, the lack of Sox9b function in zebrafish would first impair these ductal progenitors, leading to a reduced number of intrapancreatic ductal cells. The first two waves of endocrine cells, as well as the acinar cells, which originate from sox9b negative tissue, would therefore not be affected in a sox9b mutant.

Loss of Sox9b function in zebrafish severely impairs the development of the intrapancreatic biliary network including the morphogenesis of the bile canaliculi. These phenotypes are more severe than those seen in liver-specific SOX9-depleted mice [23]. During mouse liver development, SOX9 expression is first detected at E10.5 in the endodermal cells lining the lumen of the liver diverticulum [23]. This expression is lost as the liver cells migrate into the septum transversum, but re-emerges at E11.5 in cells that form the ductal plate. The Alfp:Cire line that was used to recombine the Sox9 locus becomes active at E11.5, thus likely only inhibiting the second phase of SOX9 expression. Therefore, the phenotypic differences between the zebrafish sox9b mutant and the mouse model might be related to the consequences of an earlier depletion in Sox9b in zebrafish than in mouse, illustrating the value of the zebrafish Sox9b global loss-of-function model to uncover functions of this critical transcriptional regulator. However, differences in the expression or function between sox9b (zebrafish) and Sox9 (mouse) may also explain the phenotypic differences between the two models.

Intrapancreatic biliary cells in sox9b mutant livets fail to separate from one another and remain clustered, leading to a primitive ductal network. Such defects are strikingly similar to Notch-deficient zebrafish and mouse models [6,9,20,21,41] which phenocopy the human Alagille syndrome (OMIM #118450), which itself is associated with JAGGED1 and NOTCH2 mutations. Notably, this developmental disorder is characterized by cholestasis due to a paucity of biliary ducts. Our data indicate that Sox9b interacts with Notch signaling in a positive feedback loop to regulate intrapancreatic biliary duct morphogenesis. Studies in mouse have provided possible mechanisms underlying this Sox9-Notch crosstalk: the Sox9 promoter displays ten consensus Rbpj binding sites and is a direct target of Notch signaling [9]. In addition, SOX9 modulates Notch signaling by positively regulating the expression of the Notch downstream target gene Hes1 in the liver [23] as well as in other organs such as the pancreas [25]. These mechanisms are likely to be at play in zebrafish as well, and it will be interesting to delve deeper into the complexities of this positive feedback loop.
Intriguingly, we found that the biliary cells in the proximal region of wild-type livers tend to lose Notch signaling more quickly and are more susceptible to Sox9b loss than the distal cells. Notably, the biliary cells in the proximal region form large ducts whereas the distal cells form small ducts. In sox9b mutants, loss of Sox9b function impairs Notch signaling in all biliary cells. Consequently, the mutant livers exhibit aberrant clusters of Tg(Tp1glob:H2B-mCherry)-single positive cells in their distal region, suggesting that lack of Sox9b-mediated maintenance of Notch signaling could promote the formation of large ectopic ducts in distal regions of the liver. Interestingly, it has been shown in mouse that Sox9, which is expressed in all biliary cells at E18.5, persists in small ducts but regresses from large ducts after birth [23]. Therefore, it is possible that in wild-type animals, the loss of Notch signaling in the proximal region of the liver induces the local loss of Sox9 expression, leading to the formation of large bile ducts. To begin to test this hypothesis, it will be necessary to generate a Sox9b antibody in order to examine Sox9b expression at cellular resolution. The identification of markers that distinguish large and small bile ducts would also greatly facilitate such studies.

Our data suggest that in addition to regulating biliary morphogenesis, the Notch-Sox9b module also influences the proliferation of biliary cells. In sox9b mutants, the biliary cells that turn off Notch signaling exhibit a higher proliferation rate. Recent lineage tracing studies in mouse have shown that Sox9 is expressed in liver progenitor cells that reside within the biliary ducts [4,48]. It will be interesting to determine whether zebrafish sox9b is also expressed in liver progenitor cells, whether loss of Sox9b function affects their proliferation rate, and investigate into the underlying mechanisms.

In addition to bringing new insights into our understanding of the development of the pancreaticobiliary ductal system, the analysis of zebrafish sox9b mutants should lead one to consider SOX9 as a candidate gene for human diseases associated with HPD, intrapancreatic or intrahepatic duct malformations. In particular, numerous cases of congenital non-syndromic or syndromic extrahepatic biliary atresia have been reported [49] and their causes remain unknown [50]. These conditions likely have multifactorial causes and do not display simple Mendelian inheritance. Given the expression pattern of SOX9 in mammals [24,26,28] as well as the phenotypes caused by loss of SOX9 function in zebrafish and mouse, SOX9 is therefore an interesting gene to sequence in those patients. Campomelic dysplasia has been shown to be essentially associated with homozygous mutations that are predicted to severely disrupt SOX9 protein structure and function [51]; but milder lesions could be associated with pancreaticobiliary duct malformations and contribute to the onset or severity of these malformations without necessarily impairing skeletal development.

**Materials and Methods**

**Zebrafish strains and lines**

Embryos and adult fish were raised and maintained under standard laboratory conditions [32]. The sox9b^{kh313} heterozygote was crossed with Tg(fabl:1cras-GFP)^{1.5kca4} [37], Tg(Tp1glob:GFP)^{m41} [35], Tg(Tp1glob:H2B-mCherry)^{1.5kca4} [34], TgBAC(neurod:GFP)^{58} and genotyped according to the TILLING center protocol with Aciu or Sfci (http://labs.ihcrc.org/moons/TILLING_Mutants/sox9b/allele_1.html). We also used Tg(hsp70l:Gal4)^{1.5kca4} and Tg(UASmyc-Notch1a-intra)^{3.8x} [43] hemizygous or double hemizygous fish.

**In situ hybridization and immunohistochemistry**

Whole-mount in situ hybridizations were performed as described previously [54] using sox9b [30] and sox17 [45] probes. Animals were photographed with a Zeiss Axioplan using an Axioscam digital camera. Immunohistochemistry on whole-mount animals or cryosections was performed as previously described [16], using the following antibodies: chicken polyclonal anti-GFP (1:1000; Aves Labs, Tigard, OR, USA), rabbit polyclonal anti-Prox1 (1:1000; Chemicon, Billericia, MA, USA), mouse monoclonal 2F11 (1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-dsRed (1:500; Clontech, Mountain View, CA, USA), rabbit polyclonal anti-ABCB11/BSEP (1:1000; Kamiya Biomedical), mouse monoclonal anti-Alcian/ZN9 (1:20; ZIRC), rabbit polyclonal anti-elastase (1:200; Millipore AB1216) and fluorescein conjugated Alexa antibodies (1:250; Molecular Probes, Carlsbad, CA, USA). Samples were imaged on a Zeiss Pascal confocal microscope. The width of the bile ducts was measured using the “local thickness” function in Fiji software.

**Heat-shock experiment and chemical inhibitor treatment**

Heat-shock treatments of Tg(hsp70l:Gal4)^{1.5kca4} and Tg(UASmyc-Notch1a-intra)^{3.8x} were performed at 38°C as described [16]. To inhibit Notch signaling, larvae were treated with 20 μM or 50 μM DAPT (Sigma) in egg water [42]. Control larvae from the same batch were treated with 0.4% DMSO in egg water. Statistical analyses were performed using Student’s two-tailed t-test.

**Histology**

Five-months old zebrafish (one wild-type and one mutant) were euthanized and their digestive systems were dissected and fixed with formalin at 4°C. The samples were embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin.
BODIPY assay

6–7 dpf larvae were fed with BODIPY C2.0 or BODIPY C5.0 as described in [40] for 6–8 hours before being mounted and imaged live. The larvae were subsequently genotyped and 9 wild-type and 9 sox9b mutant animals were analyzed.

EdU cell cycle analysis

To assess the proliferation of the intrahepatic biliary cells, wild-type and sox9b mutant larvae were incubated in 7 μM EdU dissolved in egg water during the stages indicated. Control larvae collected from the same batch were treated with 1.7% DMSO. Animals were fixed after incubation and processed using the Click-iT EdU Imaging Kit (Invitrogen). Quantification of EdU incorporation was conducted using the Cell Counter plug-in in ImageJ.

Supporting Information

Figure S1 sox5a in situ hybridization in wild-type and sox9b mutant larvae at 80 hpf. sox5a does not appear to be expressed in endodermal tissues in wild-type or sox9b mutant animals. Dorsal views, anterior (A) to the left. (TIF)

Figure S2 Time course analyses of the pancreas (A–F) and liver (G–L) in wild-type and sox9b mutant larvae at 2, 3, and 4 weeks. (A–F) Tg(Tp1bglob:H2B-mCherry);TgBAC(neurod:GFP) double transgenic fish from sox9b heterozygote crosses were raised together and 40 of them were fixed and genotyped at each time point. The pancreas, along with the gut, of 6 to 11 wild-type or sox9b homozygous mutant fish were dissected, stained with anti-dsRed (red), anti-GFP (green) and anti-Elastase (blue) antibodies and mounted for confocal imaging. Whereas wild-type pancreata showed complex intrapancreatic duct networks at 2 and 3 weeks of age with several main pancreatic ducts (A, C), sox9b mutant pancreata failed to expand and ductal cells stayed in clusters (B, D). At 4 weeks of age, the morphological differences between wild-type and sox9b mutant pancreata were even more obvious: wild-type pancreata spread over the gut starting to form lobes whereas sox9b mutant pancreata were still primitive in appearance (E, F). The defect in pancreatic growth in sox9b mutant fish is associated with a global growth retardation of the fish as indicated by the smaller size of the fish and the frequent occurrence of an unlooped gut at 4 weeks (data not shown). In addition to pancreatic duct morphological defects, sox9b mutant fish showed a deficiency in secondary islet formation as assessed by TgBAC(neurod:GFP) expression (arrowheads in B’, D’, F’). However, at the equivalent stages, wild-type fish exhibited multiple clusters of TgBAC(neurod:GFP)-positive cells along the intrapancreatic ducts (arrowheads in A’, C’). All images are projections of confocal z-stacks. Ventral views, anterior to the top right. Dashed lines delineate the pancreas. Scale bars, 100 μm. (G–L) Wild-type and mutant larvae were sorted at 5 dpf based on their intrahepatic ductal system phenotypes (as assessed by the pattern of Tg(Tp1bglob:GFP) expression) and raised separately. At 2 (G, J), 3 (H, K), and 4 weeks (I, L) of age, 2 wild-type and 2 mutant fish were fixed for immunostaining. Cryosections were stained with 2F11 (red) and anti-GFP (green) antibodies. In the liver, whereas the intrahepatic biliary network continued to expand between 2 and 4 weeks in wild-types (G–I), the biliary cells in sox9b mutants remained clustered and never assumed normal morphogenesis (J–L). Moreover, the expression of Tg(Tp1bglob:GFP) largely overlapped with 2F11 labeling in wild-type livers (G–I). In contrast, in sox9b mutant livers, we observed an increasing number of cells that were labeled by 2F11, but did not express Tg(Tp1bglob:GFP) (J–L). These cells formed large clusters by 4 weeks of age (L). All images are projections of confocal z-stacks. Sagittal sections, anterior to the top. Dashed lines in (G–L) delineate the liver. Li, liver; Pa, pancreas. Scale bar, 20 μm. (TIF)

Figure S3 sox9b mutants show defects in bile secretion and transport as assessed by BODIPY-FL analog feeding. (A–B) Fluorescent micrographs of 7 dpf live wild-type (A) and sox9b mutant (B) larvae after BODIPY feeding showing lack of filling of the gallbladder (arrowhead) in sox9b mutants (B). Lateral views, anterior (A) to the left. (C–F) Confocal images of Tg(Tp1bglob:H2B-mCherry) wild-type (upper panel) and sox9b mutant (lower panel) larvae showing morphological and functional defects of both intrahepatic (D) and intrapancreatic (F) ductal networks compared to wild-type (C and E). In the mutants, both intrahepatic and intrapancreatic ducts appear to be dilated (D and F). Fluids (bile or pancreatic juice) also appear to accumulate in the pancreatic tail (arrows, F). Dashed squares represent areas shown in higher magnification for intrahepatic (C’–D’) and intrapancreatic (E’–F’) ducts in wild-types (upper panel) and sox9b mutants (lower panel). sox9b mutants showed defects in bile canaliculi (comparing arrowheads in C’, D’) and terminal pancreatic ducts (comparing arrowheads in E’, F’). 9 larvae were analyzed for each genotype. (C–F) All images are projections of confocal z-stacks. (C–F) Lateral views, anterior (A) to the left. Dashed lines in E and F outline the pancreas, g; gut; Li, liver. Scale bars, 20 μm. (TIF)

Figure S4 The intrahepatic biliary cells in sox9b mutants are more susceptible to Notch signaling inhibition. (A–D) Confocal images of livers in Tg(Tp1bglob:VenusPest); Tg(Tp1bglob:H2B-mCherry) wild-type (A, C) and sox9b mutant (B, D) larvae treated with DMSO (A–B) or 50 μM DAPT (C–D) from 106 to 154 hpf. DAPT treatment caused an increase in the relative proportion of Tg(Tp1bglob:H2B-mCherry)-single positive cells in all the animals, yet sox9b mutants exhibited a more severe increase compared to wild-type larvae. In DAPT-treated wild-type (C), loss of Notch activity was more prominent in the region proximal to the extrapancreatic duct (p, left side of yellow line), whereas the distal biliary cells still maintained Tg(Tp1bglob:VenusPest) expression (d, right side of the yellow line). (A–D) All images are projections of confocal z-stacks. Ventral views, anterior (A) to the top. Dashed lines outline the liver. Scale bar, 50 μm. (E) Percentages (average±SEM) of Tg(Tp1bglob:H2B-mCherry)-single positive cells relative to the total number of Tg(Tp1bglob:H2B-mCherry)-expressing cells. 10 DMSO control and 14 DAPT-treated larvae were analyzed for each genotype. Asterisks indicate statistical significance compared to equally-treated wild-type larvae: ***, p<0.005; ******, p<0.000005. (TIF)

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Author Contributions

Conceived and designed the experiments: MD CY DYRS. Performed the experiments: MD CY DS LP TPM. Analyzed the data: MD CY DYRS. Contributed reagents/materials/analysis tools: NN JDC SAF CBM. Wrote the paper: MD CY DYRS. Commented on the manuscript: DS NN JDC SAF CBM.
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