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Résumé

Le foie est un organe vital qui exerce plus de 500 fonctions différentes. Les hépatocytes, qui constituent la majeure partie du foie, assurent l'essentiel des fonctions métaboliques et sécrètent la bile. Celle-ci est drainée vers l'intestin par les canaux biliaires bordés par des cholangiocytes ou cellules biliaires.

Les hépatocytes et les cholangiocytes sont issus de précurseurs embryonnaires communs appelés hépatoblastes. Les hépatoblastes se situant à proximité de la veine porte se différencient en cellules biliaires, constituant une couche unicellulaire de cellules entourant le mésenchyme portal, appelée plaque ductale. Les canaux biliaires se développent à partir de cette plaque ductale en formant en premier lieu des structures ductales primitives transitoires. Ces structures transitoires sont asymétriques et forment des lumières délimitées du côté portal par des cellules biliaires de la plaque ductale et du côté parenchymateux par des hépatoblastes. Ces derniers se différencient ensuite en cholangiocytes, tout comme les cellules de la plaque ductale, contribuant ainsi à la formation des canaux biliaires matures.

Des travaux antérieurs au sein de notre laboratoire, ont montré que le facteur de transcription *Sry-related HMG box* (SOX) SOX9 est le marqueur de différentiation biliaire le plus précoce et qu'il régule le timing du développement des canaux biliaires. Nous avons identifié que le facteur SOX4, appartenant à la même famille de facteurs SOX, était également exprimé dans les cellules biliaires, dès le début de leur développement. Nous avons montré que les facteurs SOX4 et SOX9 coopèrent et contrôlent la différentiation et la morphogenèse des canaux biliaires. Par ailleurs, nous avons approfondi les connaissances sur le rôle de la protéine liver kinase B1 (LKB1) et de la voie de signalisation Notch durant le processus de développement biliaire.

Nos données permettent de mieux comprendre les mécanismes régulant la différentiation des hépatoblastes en cholangiocytes et la tubulogenèse des canaux biliaires. De plus, nos travaux ouvrent de nouvelles perspectives dans la compréhension des malformations des voies biliaires chez l'Homme, et dans la production de cholangiocytes *in vitro* à des fins thérapeutiques.

Table of content

Abbreviations

ADPKD	Autosomal dominant polycystic kidney disease
Alfp	Albumin/α-fetoprotein
AMPK	AMP-activated protein kinase
APC	Adenomatosis Poliposis Coli
ARC	Arthrogryposis-renal dysfunction and cholestasis syndrome
ARPKD	Autosomal recessive polycystic kidney disease
Bd	Bile duct
BMP	Bone morphogenic protein
BRSK	BR serine/threonine-protein kinase
C/EBP	CCAAT/Enhancer Binding Protein
CCA	Cholangiocarcinoma
ChIP	Chromatin immunoprecipitation
CK	Cytokeratin
CPS1	Carbamoyl phosphate synthase 1
Cre	Cyclization recombinase
CV	Central vein
DBA	Dolichos-biflorus agglutinin
Dp	Ductal plate
Ecad	E-cadherin
ECM	Extracellular matrix
EHBD	Extrahepatic bile ducts
FGF	Fibroblast growth factors
FGFR	FGF receptor
FoxA	Forkhead box A
Foxm1b	Forkhead Box m1b
Grhl2	Grainyhead-like 2
GS	Glutamine synthetase
GSEA	Gene Set Enrichment Analysis
HCC	Hepatocellular carcinoma
HDGF	Hepatoma-derived growth factor
Hes1	Hairy and Enhancer of Split-1
hESC	Human embryonic stem cells
HGF	Hepatocyte growth factors
Hhex	Hematopoietically expressed homeobox

HMG	High mobility group
HNF	Hepatocyte nuclear factor
IHBD	Intrahepatic bile ducts
ISH	In situ hybridization
Jag1	Jagged1
Lam	Laminin
LEFT	Liver-enriched transcription factors
LKB1	Liver kinase B1
LRH1	Liver Receptor Homolog 1
MARK	MAP/microtubule affinity regulating kinase
Mes	Mesenchyme
Mir	MicroRNA
mTOR	Mammalian target-of-rapamycin
Muc	Mucin 1
Nf2	Merlin/Neurofibromin 2
NICD	Notch intracellular domain
OC	Onecut
OPN	Osteopontin
OSM	Oncostatin M
PC	Polycystin
PDS	Primitive ductal structures
PDX1	Pancreatic and duodenal homeobox 1
Pkhd1	Polycystic kidney and hepatic disease 1
Prox1	Prospero-homeobox protein 1
RBPJκ	Recombination signal binding protein for immunoglobulin kappa J
Sall4	Sal-like 4
Smad	SMA- and MAD-related protein
SOX	SRY-related High Mobility Group box
Sry	Sex-determining region of the Y chromosome
TAD	Transactivation domain
Tbx3	T-box transcription factor 3
TEAD	TEA domain transcription factor
TGFβ	Transforming growth factor beta
TRD	Transrepression domain
TβRII	TGFβ receptor type II

1. INTRODUCTION

1.1. The liver and hepatobiliary system

1.1.1. Liver function and architecture

The liver plays a wide range of homeostatic functions and can be considered as an endocrine and exocrine organ. It is essential for glycogen storage, drug detoxification, regulation of cholesterol synthesis and transport, urea metabolism and production of plasma proteins such as albumin and apolipoproteins. It also secretes several hormones such as Insulin-like growth factors, angiotensinogen and thrombopoietin; exocrine functions mainly consist in the production and secretion of bile into the intestine.

Only a limited number of cell types are required to carry out the numerous liver functions. Hepatocytes occupy 80% of the liver volume and exert the major metabolic activities of the liver (Blouin et al., 1977). The liver is also composed of cholangiocytes (biliary cells lining the bile ducts), endothelial cells (lining blood vessels and sinusoids), Kupffer cells (macrophages), stellate cells and pit cells (natural killer cells).

From an architectural point of view, the liver is divided into basic functional units called lobules. They are roughly hexagonal and display a honeycomb-like shape. Within the lobules, hepatocyte cords are lined by sinusoidal capillaries that radiate toward a central vein. A portal triad composed of the portal vein, the hepatic artery and a bile duct is located at each corner of the lobule (Fig. 1). The portal vein supplies blood from the intestine to the liver, while oxygenated blood is provided by the hepatic artery. Lobules are irrigated through the sinusoids and blood is collected at the central vein and further drained to the vena cava. Exchange of solutes between the sinusoids and hepatocytes occurs via the basolateral pole of hepatocytes and the fenestrated sinusoidal endothelial cells (Fig. 1).

Hepatocytes produce and excrete bile at their apical pole. Bile transits through the canaliculi, bile ducts and extrahepatic ducts prior to being delivered to the

duodenum (see also § 1.1.2 Function and organization of the hepatobiliary system). Therefore, blood and bile flows are anti-parallel in the liver (Fig. 1).

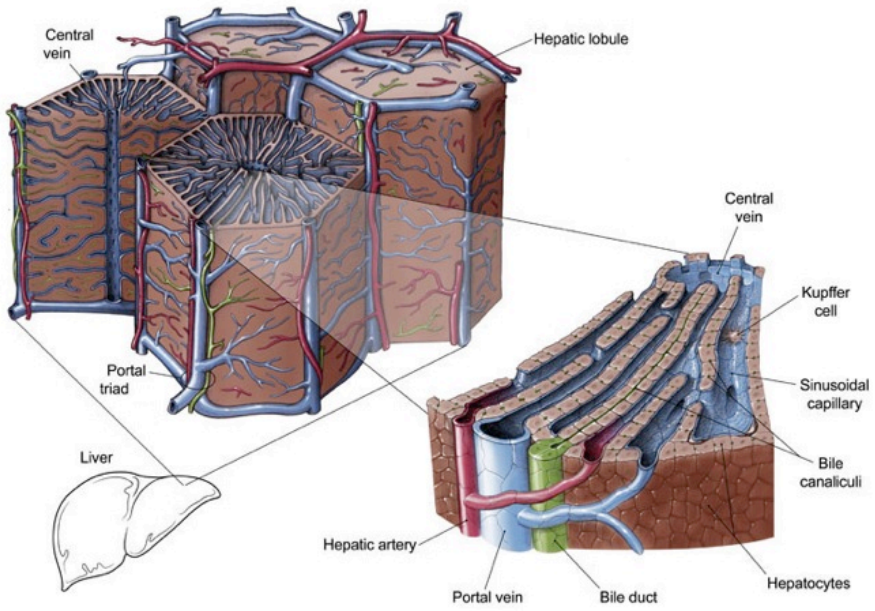

Figure 1. Architecture of the liver (adapted from http://illuminationstudios.com). Schematic representation of the structure and organization of the liver lobules.

1.1.2. Function and organization of the hepatobiliary system

Bile is composed of water, conjugated bile salts and bilirubin, lipids (cholesterol, fatty acids and lecithin) and ions. The amphipathic properties of bile salts enable formation of micelles and promote digestion and absorption of dietary lipids. A high proportion of bile salts is recycled from the intestine to the liver (enterohepatic cycle). Bile is also important for absorption of fat-soluble substances, such as vitamins A, D, E and K, and for bilirubin and cholesterol excretion. It also displays bactericidal properties and regulates the composition of gut microbiota.

Bile is first collected in canaliculi delineated by the apical poles of adjacent hepatocytes. Leakage of bile is prevented by tight junctions between neighboring hepatocytes. Subsequently, bile flows via the canals of Hering to intralobular biliary ductules and next to the intrahepatic bile ducts. Canals of Hering are lined on one side by hepatocytes and on the other by cholangiocytes, while intralobular ductules

and intrahepatic bile ducts are delineated exclusively by cholangiocytes (Roskams et al., 2004) (Fig. 2A). Cholangiocytes modulate bile composition by absorption of solutes and secretion of bicarbonate and water. Although cholangiocytes represent only 3-5% of the liver cell population, they produce up to 40% of total bile volume (Masyuk and LaRusso, 2006a) (Fig. 2B).

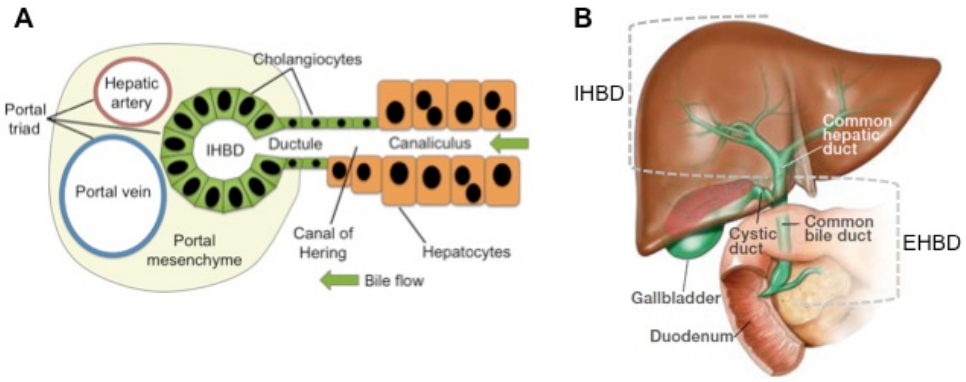

Figure 2. The hepatobiliary system. **A.** Schematic representation of the intrahepatic bile flow. **B.** Representation of the intra- and extra-hepatic biliary tract. IHBD, intrahepatic bile ducts; EHBD, extrahepatic bile ducts.

The intrahepatic bile ducts run parallel with the branches of the portal vein and hepatic artery and constitute the “intrahepatic biliary tree”: small bile ducts merge to constitute larger ducts which coalesce to form hepatic ducts. The hepatic ducts – one per lobe – fuse at the hilum to form the common hepatic duct, the first portion of the extrahepatic biliary tract (Fig. 2B).

The cystic duct drains bile to and from the gallbladder, and fuses with the common hepatic duct, giving rise to the common bile duct. The latter then merges with the pancreatic duct to enter in the duodenum, forming the ampulla of Vater (Fig. 2B). Bile is continuously produced by the liver and transported to the gallbladder where it is stored. After food intake, gallbladder contraction and relaxation of the sphincter of Oddi allow bile to be excreted into the duodenum.

1.2. Development of the liver: overview

The liver derives from the endoderm; hepatocytes and cholangiocytes, the main epithelial cell types of the liver, originate from common embryonic precursors called hepatoblasts. Here, I will describe how the endoderm gives rise to the hepatoblasts, and summarize how the latter further differentiate to hepatocytes. Development of the cholangiocytes and bile ducts, which constitutes the focus of the present thesis, is described in a separate section.

1.2.1. Development of hepatoblasts from the endoderm

After gastrulation, liver precursor cells are located in three distinct domains of the anterior endoderm (Tremblay and Zaret, 2005; Zaret, 2008). During foregut closure, these domains migrate to fuse and generate a single prehepatic domain adjacent to the cardiac mesoderm and the septum transversum.

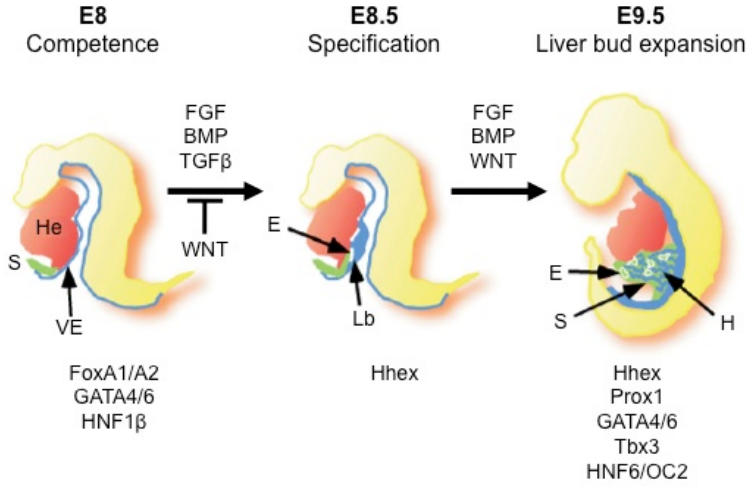

Figure 3. Onset of liver development (adapted from Zhao and Duncan, 2005). Illustration of initial steps of liver development with key transcription factors and signaling pathways involved at specific stages. He, heart; S, septum transversum; VE, ventral endoderm; E, endothelial cells; Lb, liver bud; H, hepatoblasts.

At this time, around embryonic day 8.25 (E8.25) in the mouse, cells of the prehepatic domain start expressing the transcription factors Forkhead box (Fox) A and GATA4. These factors bind to the promoter of liver-specific genes and

decompact their chromatin. The prehepatic cells are then considered "competent". They do not yet express hepatic genes but are primed to respond to extracellular signals that will induce expression of liver-specific genes (Bossard and Zaret, 1998; Cirillo et al., 2002; Gualdi et al., 1996; Lee et al., 2005; Lemaigre, 2009). Hepatocyte Nuclear Factor 1 β (HNF1β) stimulates expression of FoxA1 and FoxA2 in the prehepatic domain, and is essential for its competence (Lokmane et al., 2008). Therefore, FoxA, GATA and HNF1β factors constitute the regulatory network of hepatic competence (Fig. 3).

Shortly after acquisition of competence, at E8.5, hepatic precursor cells begin to express albumin, transthyretin, α-fetoprotein and HNF4α. This step is called "specification" and is the first molecular evidence of liver development. Hepatic specification is induced by extracellular signals arising from the adjacent cardiac mesenchyme and septum transversum. Fibroblast growth factors (FGF) are secreted by the cardiac mesoderm and induce liver-specific genes in competent hepatic progenitors (Calmont et al., 2006; Gualdi et al., 1996; Jung et al., 1999). However, FGF signaling is not sufficient for hepatic specification. Bone morphogenic protein (BMP)-2 and BMP-4 produced by the septum transversum, are also required for liver specification (Rossi et al., 2001). In addition, Transforming growth factor beta (TGFβ) signaling is necessary for hepatic precursor cells to retain their competency and to prevent them from inappropriate differentiation into pancreatic progenitors (Wandzioch and Zaret, 2009), while Wnt signaling must be repressed in the anterior endoderm to allow initiation of hepatic specification (McLin et al., 2007).

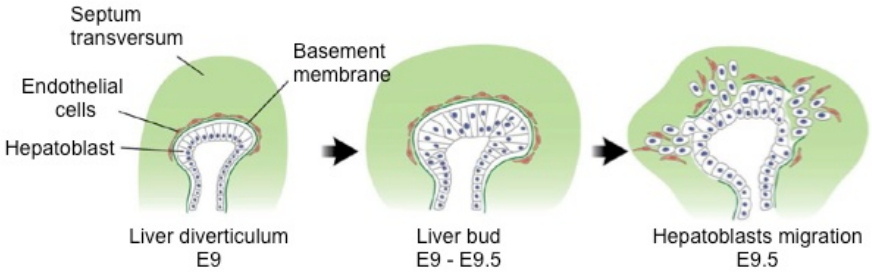

Figure 4. Liver budding. Schematic representation of the liver diverticulum, liver bud and invasion of the septum transversum by hepatoblasts.

The specified hepatic precursor cells are called hepatoblasts. Around E9, the hepatoblasts line an evagination of the endoderm (liver diverticulum). They become columnar and undergo a transition – dependent on the *Hematopoietically Expressed Homeobox* factor Hhex – to a pseudostratified epithelium (Bort et al.,

2006). The hepatoblats proliferate and subsequently form a tissue bud delineated by a basal membrane. Slightly later the hepatoblasts migrate through this basement membrane and invade the septum transversum (Fig. 4). Liver budding and hepatoblast invasion require cell proliferation, migration, loss of adhesion and basement membrane remodeling. These processes are controlled by a network of genes involving Hhex, GATA-6, HNF6, Onecut 2 (OC2), T-box transcription factor 3 (Tbx3) and Prospero homeobox protein 1 (Prox1) (Lemaigre, 2009) (Fig. 3).

1.2.2. Differentiation of hepatocytes

Once the hepatoblasts have invaded the septum transversum, the embryonic liver cells must organize to establish a functional organ architecture (see § 1.1.1. Liver function and architecture). This involves proliferation and differentiation of the liver cell types, liver vascularization, extracellular matrix organization, hepatocyte maturation and biliary tree development.

After invading the septum transversum, hepatoblasts continue to proliferate in order to expand the mass of the liver bud. Several signaling pathways and growth factors arising from septum transversum, endothelial cells and hepatoblasts control this proliferation. These include Hepatocyte growth factors (HGF) and Hepatoma-derived growth factor (HDGF) as well as FGF, TGFβ and Wnt/β-catenin signaling (Berg et al., 2007; Enomoto et al., 2002; Schmidt et al., 1995; Tan et al., 2008; Weinstein et al., 2001).

Then, bipotent hepatoblasts differentiate into hepatocytes or cholangiocytes (Germain et al., 1988; Shiojiri, 1984, 1997; Spagnoli et al., 1998). Once committed to the hepatocyte lineage, hepatocyte precursors gradually mature: their gene expression profile, metabolic functions and cord-like architecture are progressively acquired from E12 onwards. A dynamic transcriptional network of liver-enriched transcription factors (LETFs) controls hepatocyte maturation (Kyrmizi et al., 2006). LETFs form autoregulatory and cross-regulatory loops which evolve during hepatocyte maturation: concentration of LETFs and number of interactions increase with time resulting in a stabilization of this network. Within this network, a "core" of 6 transcription factors (HNF1α, HNF1β, FoxA2, HNF4, HNF6 and Liver receptor homolog 1 (LRH1)), which bind to the regulatory regions of each LETFs, has been identified (Kyrmizi et al., 2006; Lemaigre, 2009). LETFs control hepatocyte maturation by gradually inducing expression of genes that are essential for hepatocyte functions such as glucose, lipid and amino acid metabolism or bile

acid homeostasis. They also control the acquisition of cell polarity and morphology (Lemaigre, 2009).

Extracellular signals are required for hepatocyte maturation. Hematopoietic precursor cells which reside in fetal liver produce Oncostatin M (OSM). This cytokine stimulates expression of terminal hepatocyte differentiation markers (Kamiya et al., 1999). Moreover, HGF and Wnt ligands, secreted by endothelial cells, allow the organization of hepatocytes into cord-like structures and stimulate glycogen accumulation in hepatocytes (Matsumoto et al., 2008; Suzuki et al., 2006).

After birth, hepatocytes progressively constitute a heterogeneous cell population. Within an hepatic lobule, hepatocytes express different gene sets and exert different metabolic functions according to their distance from the portal vein and the centrolobular vein. This process is called liver zonation and is regulated by a gradient of Wnt/β-catenin signaling and by zone-specific activities of HNF4 (Benhamouche et al., 2006; Stanulovic et al., 2007).

1.2.3. The polarized architecture of hepatocytes

Hepatocytes form a polarized tissue and correct polarization of the latters is essential to ensure the metabolic functions of the liver. Hepatocytes are aligned in single-cell thick cords lined by a fenestrated sinusoidal endothelium (Fig. 5). This cell organization implies a singular polarization of hepatocytes. Indeed, hepatocytes have opposing basal surfaces that are in contact with the extracellular matrix (ECM), which separates hepatocytes from sinusoidal endothelial cells, while they are connected to neighboring hepatocytes via their lateral membranes. Hepatocytes and sinusoidal cells are separated by the space of Disse (Fig. 5). This space contains blood plasma leaked through the fenestrated sinusoids, allowing proteins and other plasma components to be absorbed by the hepatocytes. The ECM lining the space of Disse is mostly composed of fibronectin and collagen type I, as well as small amounts of collagen type III, IV, V and VI, but it is devoid of the major basement membrane component laminin (Arriazu et al., 2014; Bataller and Brenner, 2005; Desmet et al., 1995). At the lateral membrane, hepatocytes form a small apical domain delineated by tight junctions. Theses apical domains constitute the bile canalicular network, which allows transport of bile in liver parenchyma toward the canal of Hering and subsequently to the bile ducts (Fig. 5). Tight junctions create a blood-biliary barrier, and serve to separate the apical and basolateral domains to maintain hepatic polarity (Tsukita et al., 2001).

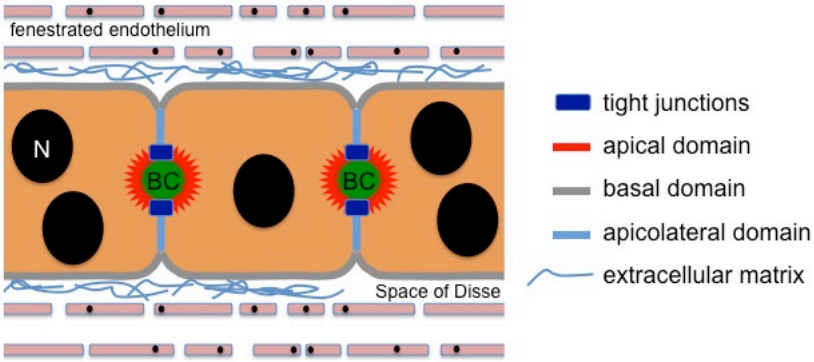

Figure 5. Singular hepatocyte polarization. Hepatocytes form an apical lumen delineated by two adjacent hepatocytes. Hepatocytes contact the blood and ECM via opposing basal membrane domains. N, nucleus; BC, bile canalicus.

Bile acids, via a cAMP-LKB1-AMPK pathway, stimulate hepatocyte polarity and canalicular network formation (Fu et al., 2010; Fu et al., 2011; Homolya et al., 2014). Moreover, it has been shown that OSM also stimulates hepatocyte polarity (Van et al., 2004; Wojtal et al., 2007). Importantly, HNF4 plays a major role during hepatocyte polarization and liver architecture notably by regulating expression of genes involved in cell junction assembly and adhesion (Battle et al., 2006; Lemaigre, 2009; Parviz et al., 2003). Maintenance of hepatocyte polarity during cell division requires a tight control of the orientation of the cleavage furrow; this orientation is tightly controlled by Par1b (Par1b induces asymmetric inheritance of plasma membrane domains via LGN-dependent mitotic spindle orientation in proliferating hepatocytes) (Slim et al., 2013).

1.3. Development of the intrahepatic biliary tree

The extra- and intrahepatic biliary tracts are contiguous and their lumen is lined by cholangiocytes. For these reasons, it has been argued for a long time that they both arise from hepatoblasts. This is not the case. The extrahepatobiliary system does not have a hepatic origin. Instead it shares a common origin with the ventral pancreas. At E8.5, extrahepatobiliary and pancreatic progenitors in the endoderm form a single and unique population of Pancreatic and duodenal homeobox 1 (PDX1) / SRY-related High Mobility Group box 17 (SOX17) positive cells, which

then segregates into a PDX1-positive ventral pancreas and a SOX17-positive biliary primordium. SOX17 is necessary and sufficient to specify a biliary fate (Spence et al., 2009). Cholangiocytes lining intrahepatic bile ducts arise from the hepatoblasts, and therefore originate from a distinct endodermal domain. As previously mentioned, hepatoblasts give rise to either hepatocytes or to cholangiocytes and are considered bipotential (Germain et al., 1988; Shiojiri, 1984, 1997; Spagnoli et al., 1998).

1.3.1. Intrahepatic biliary morphogenesis

During liver development, the earliest biliary precursor cells start to express the transcription factor SOX9 and are observed at E11.5 in the vicinity of the portal vein mesenchyme (Antoniou et al., 2009). Progressively, a growing number of hepatoblasts differentiate to SOX9-positive cholangiocyte precursors until these cells surround the branches of the portal vein and form a single-layered ring called "ductal plate" (Fig. 6). Differentiation of hepatoblasts toward biliary precursors is associated with increased E-cadherin (Ecad) expression.

At E15.5, ductal plate cells and adjacent hepatoblasts form primitive ductal structures (PDS) and mark the onset of bile duct morphogenesis. PDS are asymmetrical structures whose lumen is delineated on the portal side by SOX9-positive biliary cells and on the parenchymal side by SOX9-negative hepatoblasts (Antoniou et al., 2009). Cells on the portal side also express biliary markers (Cytokeratin 19 (CK19), HNF1β, Laminin) and repress hepatoblasts markers (HNF4). However, Osteopontin (OPN) and tight junction protein ZO-1 are found at the apical pole of all PDS cells indicating that polarization of ductal cells becomes established as soon as the lumen of the PDS is detectable (Antoniou et al., 2009) (Fig. 6).

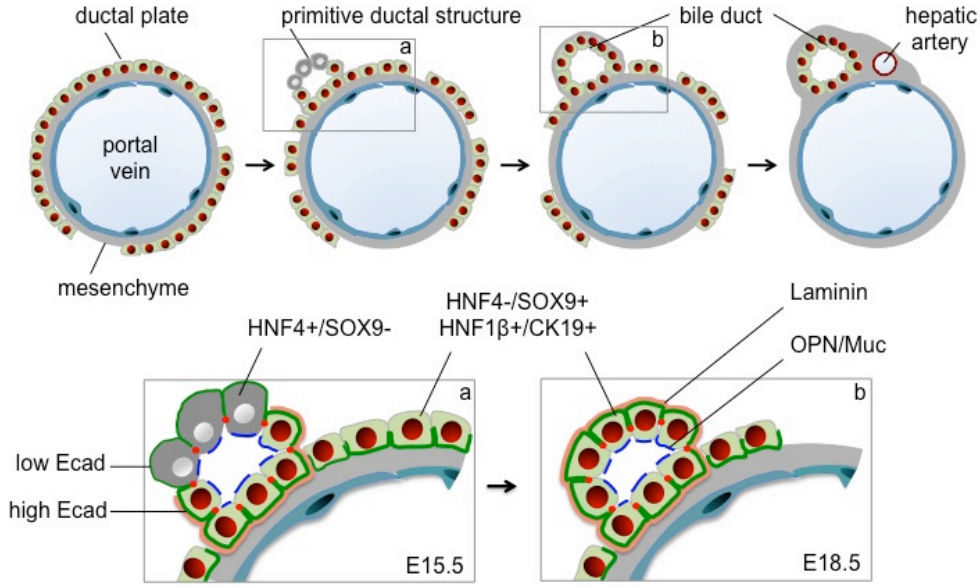

Figure 6. Schematic representation of bile duct development. Chronology of biliary morphogenesis from ductal plate formation (around E13) to mature bile duct and portal triad (early postnatal period). (a and b) Magnification of boxed biliary structures at E15.5 and E18.5. Orange line represents ECM deposition at the basal pole of cholangiocytes and blue lines represent the apical pole of cholangiocytes. Red dots illustrate tight junctions between neighboring cholangiocytes. Ecad, E-cadherin; CK19, Cytokeratin 19; OPN, Osteopontin; Muc, Mucin-1.

The asymmetry of PDS is transient and ductal hepatoblasts undergo a radial and progressive differentiation into biliary cells. At E18.5, all cells lining the ducts have acquired their typical cholangiocyte morphology (Antoniou et al., 2009). Bile ducts are surrounded by extracellular matrix (ECM) and mesenchyme.

Part of the ductal plate is not involved in duct formation. Ductal plate cells not involved in bile duct morphogenesis redifferentiate and give rise to periportal hepatocytes, and to cells lining the canals of Hering (Carpentier et al., 2011). In parallel, the portal mesenchyme expands as a result of proliferation. Branches of the hepatic artery develop postnatally in the mesenchyme, and in mice this process is dependent on bile duct development (Clotman et al., 2003; Raynaud et al., 2011b) (Fig. 6).

Bile duct morphogenesis gradually progresses along a hilum-to-periphery axis. During development, the most mature biliary structures are found close to the

hilum near the largest branches of the portal vein, while ducts at the periphery of the liver lobes mature at a later stage (Van Eyken et al., 1988). Therefore, the embryonic stages mentioned above are valid for the most mature biliary structures located near the hilum.

1.3.2. Cholangiocyte polarity

In bile ducts, cholangiocytes exhibit an apico-basal polarity with an apical pole facing the lumen and a basal pole in contact with the ECM. Tight junctions are present between neighboring cholangiocytes as shown by the expression of ZO-1 and Claudin 15 (Antoniou et al., 2009; Cheung et al., 2012; Tanimizu et al., 2012) (Fig. 6, box b).

As mentioned above, PDS cells start to establish an apico-basal polarity as soon as lumens form. This is detectable by the expression of apical markers such as OPN and Mucin1 in all ductal cells starting at E15.5. However, as shown by laminin staining, ECM deposition is detected at the basal domain of biliary cells located on the portal side of PDS whereas it is absent form the parenchymal side (Antoniou et al., 2009; Tanimizu et al., 2012) (Fig. 6, box a). This reveals a dichotomy in acquisition of the apico-basal polarity of ductal cells. As for cholangiocyte differentiation, PDS cells of the portal side are one step ahead in polarity acquisition compared to those from the parenchymal side. At the end of gestation, apico-basal polarity is established in all ductal cells and a basal lamina surrounds the bile ducts (Antoniou et al., 2009) (Fig. 6, box b).

Nowadays, little is know about the transcriptional network and signaling cues that control acquisition of apico-basal polarity in biliary cells. It has been shown that liver-specific deletion of SOX9 leads to a delay in acquisition of polarity of cholangiocytes and in deposition of laminin on the parenchymal side of developing ducts. However, it has not been clearly proved that SOX9 directly control transcription of genes involved in apico-basal polarity establishment (Antoniou et al., 2009). In addition, laminin, the major component of ECM surrounding bile ducts, is essential to establish apico-basal polarity. Indeed, hepatoblasts form cystic structures with a correct apico-basal polarity only when they are grew in three-dimensional cultures containing laminin α1. Moreover, in these cultures, blocking β1-Integrin which is a receptor of laminin in biliary cells, impairs lumen formation and disrupts apico-basal polarity as shown by mislocalization of the apical marker atypical protein kinase C (aPKC) and the basal marker α6-Integrin (Tanimizu et al., 2012; Tanimizu et al., 2007). Knowing that laminin α1 is produced

by fibroblasts localized in the portal mesenchyme, this suggests that mesenchymal cells, at least, partially initiate acquisition of apico-basal polarity of biliary cells via laminin α1 expression (Tanimizu et al., 2012).

Tight junctions are physical scaffold essential to separate the apical and basolateral domains and to maintain polarity (Tsukita et al., 2001). Mutations in Claudin-1 gene have been identified in patients suffering from syndrome associating ichthyosis and neonatal sclerosing cholangitis (NISCH syndrome). The authors report that Claudin-1 is normally expressed in tight junctions of cholangiocytes and that nonsense mutation in Claudin-1 leads to bile duct paucity (Hadj-Rabia et al., 2004). Moreover, studies in zebrafish have shown that intrahepatic biliary network does not develop normally in Claudin 15-like b mutants (Cheung et al., 2012).

Together, these data reveal that correct apico-basal polarization and correct ECM deposition are essential for bile duct development.

1.3.3. Transcriptional control of bile duct development

As mentioned above, hepatoblasts commit either to a hepatocyte lineage or to a cholangiocyte lineage. Lineage segregation is a progressive process and its timing cannot be defined with accuracy.

Segregation of cholangiocytes and hepatocytes is controlled by a complex network of transcription factors. Some of these factors also play a major role in controlling bile duct morphogenesis.

The transcription factor HNF6/ONECUT1 is a centerpiece of this network. It is partially redundant with its paralog factor ONECUT2 (OC2). HNF6 and OC2 are expressed in the anterior endoderm since E8.5. They stimulate delamination of liver bud and migration of hepatoblasts in the septum transversum (Margagliotti et al., 2007). Afterwards, they are expressed in hepatoblasts, hepatocytes and predominantly in cholangiocytes. Importantly, *Hnf6* deletion leads to an early and more pronounced commitment of hepatoblasts to the biliary lineage. Biliary morphogenesis is also affected. Ductal structures form and evolve to transient and large cysts located in the vicinity of the portal vein. Hybrid cells coexpressing hepatoblast/hepatocyte and biliary markers line these cysts. The latter rapidly collapse resulting in postnatal bile duct paucity and persistence of ductal plate remnants (Clotman et al., 2005; Clotman et al., 2002). Knockout mice for OC2 show a similar but less pronounced biliary phenotype (Clotman et al., 2005). In the

combined absence of HNF6 and OC2, the phenotype is exacerbated, revealing redundancy between these two Onecut factors. All hepatoblasts differentiate into hybrid cells and aggregate to form duct-like structures surrounded by laminin (Clotman et al., 2005). Thus, HNF6 and OC2 are required to segregate the hepatocytic and biliary lineages during hepatoblast differentiation (Fig. 7). They also control bile duct morphogenesis.

The transcription factor HNF1β is highly expressed in biliary precursor cells and liver-specific deletion of *Hnf1β* mostly phenocopy *Hnf6* deletion (Coffinier et al., 2002). In rare case, humans with heterozygous mutations in HNF1β display bile duct paucity (Roelandt et al., 2012). Moreover, HNF6 binds to the HNF1β gene promoter and controls its expression in fetal livers (Clotman et al., 2002). Thus, an HNF6/HNF1β cascade controls cholangiocyte differentiation and bile duct morphogenesis (Fig. 7).

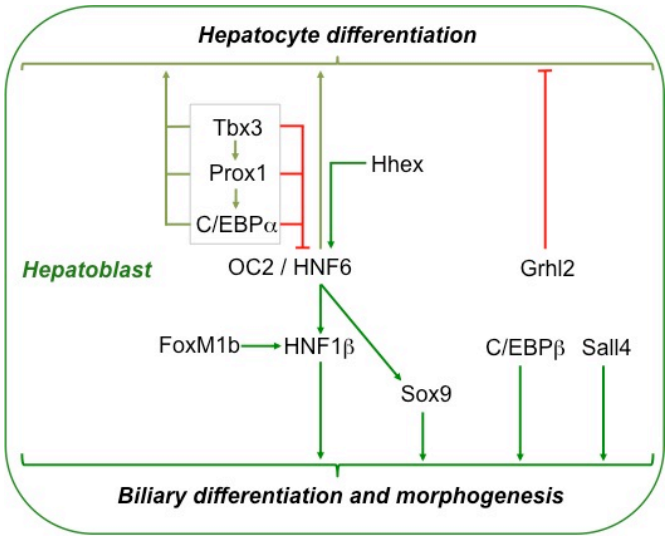

Figure 7: Schematic overview of the transcriptional network controlling hepatobiliary lineage commitment

In mouse liver, deletion of *Hnf6* reduces expression SOX9 (Antoniou et al., 2009; Laudadio et al., 2012). SOX9 has been shown to be the most specific and earliest marker of biliary cells in developing liver. Moreover, SOX9 controls the timing of bile duct morphogenesis as shown by the transient persistence of asymmetrical bile duct structures in *Sox9*-inactivated livers (Antoniou et al., 2009). Similarly, zebrafish harboring a loss-of-function mutation of the *Sox9b* gene fail to generate

normal bile ducts. However, unlike in the mouse model, biliary morphogenesis does not recover in zebrafish *Sox9b* mutants (Delous et al., 2012; Manfroid et al., 2012). Thus, SOX9 is required for normal bile duct development (Fig. 7).

Liver-specific inactivation of *Hhex* leads to formation of cystic structures composed of hybrid cells displaying hepatocyte and cholangiocyte features. Loss of *Hhex* is associated with a strong reduction of HNF6 and HNF1β expression (Hunter et al., 2007). In *CCAAT/enhancer binding protein α (C/EBPα)*-depleted livers, hybrid cells and cystic structures are also found, but in this case the phenotype is associated with overexpression of HNF6 and HNF1β (Akai et al., 2014; Tomizawa et al., 1998; Yamasaki et al., 2006). It has been shown that C/EBPα directly inhibits *Hnf6* transcription in the liver (Rastegar et al., 2000; Takayama et al., 2014). Moreover, in *Tbx3*-deficient mice, hepatoblasts preferentially commit to the cholangiocyte lineage at an early step of liver development. Biliary fate commitment is associated with downregulation of C/EBPα and overexpression of HNF6, HNF1β and SOX9 (Ludtke et al., 2009; Suzuki et al., 2008). Similarly, in *Prox1*-deficient livers, excessive cholangiocyte differentiation is observed, resulting in premature bile duct morphogenesis and biliary hyperplasia. Ectopic ductular structures develop and are lined by hybrid cells expressing hepatocyte and cholangiocyte markers. *Prox1* deletion also leads to reduced expression of C/EBPα and up-regulation of HNF6, HNF1β and SOX9 (Seth et al., 2014). The loss of *Tbx3* affects Prox1 expression while Tbx3 expression is maintained in *Prox1* knockout liver. Thus, Tbx3 is located upstream of Prox1 (Ludtke et al., 2009; Seth et al., 2014). Taken together, these data suggest that an interconnected network composed of Hhex, Tbx3, Prox1 and C/EBPα exert a tight control on HNF6 expression in order to determine normal hepatobiliary segregation (Fig. 7).

Other transcription factors control hepatoblast fate into hepatocytes or cholangiocytes. Overexpression of Sall4 in purified hepatoblasts induces expression of biliary markers and represses expression of hepatocyte genes. Moreover, hepatoblasts overexpressing Sall4 constitute larger and excessively branched ductal structures when cultured in collagen gel (Oikawa et al., 2009). Similarly, gain- and loss-of function experiments in human embryonic stem cells (hESC)-derived hepatoblast-like cells demonstrate that C/EBPβ, unlike C/EBPα, promotes cholangiocyte differentiation (Takayama et al., 2014). Moreover, Grainyhead-like 2 (Grhl2) inhibits hepatocyte differentiation while Forkhead Box m1b (Foxm1b) is critical for cholangiocyte differentiation via regulation of HNF1β (Krupczak-Hollis et al., 2004; Tanimizu et al., 2013) (Fig. 7).

Figure 7 illustrates how transcription factors interact. Surprisingly little is known about the gene targets of these factors. In zebrafish livers, the *hnf6/hnf1β* cascade controls the expression of the vacuolar sorting protein VPS33b, and *vps33b*-deficient larvae show biliary defects as reflected in the reduction of interconnecting and terminal ducts (Matthews et al., 2005) (Fig. 7). The ortholog of this gene has been found mutated in humans affected with Arthrogryposis-Renal Dysfunction and Cholestasis (ARC) syndrome which is characterized by bile duct paucity (Gissen et al., 2004). Other genes targeted by the transcription factor network are signaling mediators; these are described in the next section.

Along the same lines, post-transcriptional regulation of the transcription factors involved in hepatobiliary segregation is poorly characterized. HNF6 directly controls expression of the microRNA miR-122 in hepatoblasts. This regulation is reciprocal as miR-122 induces HNF6 expression, revealing a positive feedback loop (Laudadio et al., 2012). Preliminary data from the laboratory suggest that miR-122 represses expression of a microRNA that targets the HNF6 mRNA. MiR-122 mainly controls hepatocyte differentiation in an HNF6-dependent way. Conversely, overexpression of miR-122 also induces transient biliary hyperplasia and acceleration of SOX9 expression. How overexpression of miR-122 affects biliary development is not known (Laudadio et al., 2012).

1.3.4. Signaling pathways controlling bile duct development

Differentiation of biliary precursors, formation of the ductal plate and maturation of cholangiocytes occur in the vicinity of the portal mesenchyme, suggesting that paracrine signals induce biliary development (Shiojiri and Koike, 1997). Several signaling pathways were shown to exert such role during bile duct development.

1.3.4.1. TGFβ signaling pathway in bile duct development

During early liver development, TGFβ signaling stimulates hepatoblast proliferation. Dual haploinsufficiency for Smad2 and Smad3 – two mediators of the TGFβ pathway – causes liver hypoplasia (Weinstein et al., 2001). Later, TGFβ signaling drives hepatoblasts to the biliary fate. Around E12.5, when hepatobiliary segregation begins and prior formation of the ductal plate, a gradient of Activin/TGFβ signaling emanating from the portal mesenchyme shapes biliary differentiation. Indeed, activity of Activin/TGFβ signaling is high in the vicinity of the portal vein, where hepatoblasts will differentiate into cholangiocytes, and lower in

liver parenchyma, where hepatoblasts will commit to a hepatocyte fate (Clotman et al., 2005). Moreover, stimulating the Activin/TGFβ signaling pathway in E12.5 liver explants, cultured hepatoblasts or hESC-derived hepatoblast-like cells, induces cholangiocyte differentiation as shown by an increased expression of biliary markers and a repression of hepatoblast/hepatocyte markers (Antoniou et al., 2009; Clotman et al., 2005; Seth et al., 2014; Takayama et al., 2014). Conversely, blocking TGFβ signaling in E10.5 embryos by injecting pregnant mice with anti-TGFβ neutralizing antibodies or chemically inhibiting TGFβ signaling pathway in hESC-derived hepatoblast-like cells prevent biliary differentiation and morphogenesis (Clotman et al., 2005; Takayama et al., 2014). Taken together, these data demonstrate that TGFβ signaling is required for biliary differentiation and morphogenesis (Fig. 8).

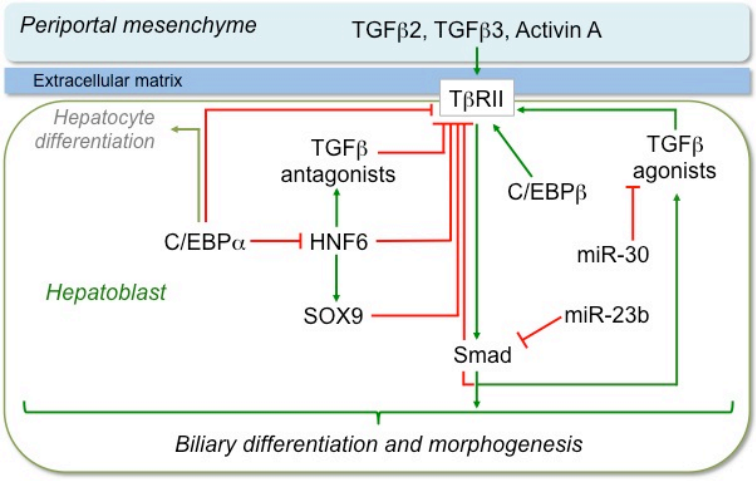

Figure 8: Schematic overview of gene interactions regulating TGFβ signaling pathway during differentiation of hepatoblasts to cholangiocytes.

Antoniou *et al.* have shown that the portal mesenchyme is the major source of TGFβ. Indeed, at E15.5, TGFβ2 and TGFβ3 mRNAs are predominantly detected in periportal mesenchymal cells while TGFβ2 and TGFβ3 proteins bind to the single-layered ductal plate cells. These cells also express the TGFβ receptor type II (TβRII). However, in PDS, TGFβ ligands and TβRII are absent from biliary cells on the portal side while they are detected in the hepatoblasts on the parenchymal side. In mature ducts, all cholangiocytes are negative for TβRII (Antoniou et al., 2009). Interestingly, delayed bile duct morphogenesis in *Sox9*-deleted livers is

associated with abnormal persistence of TβRII on the portal side of PDS. Moreover, in cultured hepatoblasts, addition of TGFβ ligands induces cholangiocyte differentiation but also TβRII repression (Antoniou et al., 2009). Taken together, these data suggest that TGFβ signaling is sequentially required for biliary differentiation on the portal side and then on the parenchymal side of PDS. When ductal cells have differentiated to cholangiocytes, TGFβ must repress its own receptor by a negative feedback loop (Raynaud et al., 2011a) (Fig. 8).

The dynamic role of the TGFβ pathway in bile duct development suggests that it is accurately and robustly controlled. The severe biliary phenotype observed in HNF6- and double HNF6/OC2-knockout mice is associated with deregulation of Activin/TGFβ signaling pathway. In the absence of the Onecut factors, Activin/TGFβ activity is higher and its gradient is misshapen. Indeed, contrary to wild-type mice where Activin/TGFβ activity is restricted to the vicinity of the portal vein, Activin/TGFβ signaling pathway extends into the liver parenchyma in knockout mice (Clotman et al., 2005). Thus, the biliary differentiation program induced by TGFβ signaling overlaps with the parenchymal program of hepatocyte differentiation and maturation. This results in pseudoglandular structures consisting of hybrid cells throughout the liver parenchyma. HNF6 and OC2 most likely control the gradient of Activin/TGFβ signaling by repressing TβRII and by inducing expression of α2-macroglobulin and follistatin, which are TGFβ and activin antagonists, respectively (Clotman et al., 2005; Plumb-Rudewiez et al., 2004). Moreover, in *Prox1*-deficient liver, HNF6 expression and TGFβ activity are increased (Seth et al., 2014). However, there is no evidence for direct repression of TβRII by HNF6 (Plumb-Rudewiez et al., 2004; Takayama et al., 2014) (Fig. 8).

Recently, it has been shown that C/EBP factors are key regulators of TGFβ activity. C/EBPβ directly induces TβRII expression and thus enables response to TGFβ. This results in cholangiocyte differentiation of hESC-derived hepatoblast-like cells overexpressing C/EBPβ. Conversely, C/EBPα directly represses TβRII expression, leading to hepatocytic differentiation of hESC-derived hepatoblast-like cells (Takayama et al., 2014) (Fig. 8).

TGFβ signaling is also regulated by microRNAs. Inhibition of *miR-23b* in cultured hepatoblasts increases Smad expression and is associated with an increase in biliary gene expression. Conversely, repression of Smads by overexpression of miR-23b inhibits formation of ductal-like structures by hepatoblast cells plated on matrigel (Rogler et al., 2009). Moreover, miR-30a and miR-30c, two microRNAs

which are specifically expressed in biliary cells and required for normal bile duct morphogenesis in zebrafish, modulate expression of Activin A, a ligand of Activin/TGFβ signaling pathway (Hand et al., 2009) (Fig. 8).

1.3.4.2. Notch signaling pathway in bile duct development

In humans, mutations in the Notch ligand Jagged1 (Jag1) or in the Notch2 receptor cause Alagille syndrome, an autosomal-dominant disorder associated with multiorgan abnormalities which include bile duct paucity and cholestasis (Alagille et al., 1987; Emerick et al., 1999; Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997). These observations point to a critical role of Notch in bile duct development. Notch signaling is involved at several stages of bile duct development, namely segregation of the hepatobiliary lineages and morphogenesis of the ducts.

Deletion of RBPJκ – the key effector protein of Notch signaling – before the onset of biliary development results in a strongly reduced numbers of biliary cells, and eventually in the absence of bile ducts (Zong et al., 2009). Moreover, zebrafish larvae deficient in Jagged proteins or combining Jagged and Notch receptor deficiency do not form biliary cells, and their hepatocytes express biliary markers (Lorent et al., 2004). These data argue that Notch signaling directs bipotent hepatoblasts toward a biliary fate. Indeed, activation of Notch signaling in cultured hepatoblasts inhibits hepatocyte gene expression and induces expression of biliary markers (Kodama et al., 2004; Tanimizu and Miyajima, 2004). This was further confirmed by *in vivo* gain-of-function experiments. Parenchymal hepatoblasts, normally fated to the hepatocyte lineage, differentiate into cholangiocytes when they constitutively express NICD, the activated form of Notch (Jeliazkova et al., 2013; Lorent et al., 2004; Tchorz et al., 2009; Zong et al., 2009). Therefore, Notch can convert hepatoblasts towards the biliary lineage (Fig. 9).

Conditional deletion of RBPJκ in mouse livers at distinct developmental stages, or administration of pharmacological Notch inhibitors at distinct phases of zebrafish liver development demonstrated that Notch controls biliary development by sequentially regulating cholangiocyte fate commitment and bile duct morphogenesis (Lorent et al., 2010; Zong et al., 2009) (Fig. 9). Deletion of components of the Notch pathway during late stages of bile duct development results in bile duct paucity, even though initial cholangiocyte differentiation has not been affected (Geisler et al., 2008; Jeliazkova et al., 2013; Zong et al., 2009). Moreover, bile duct structures are found in the parenchyma when hepatoblasts constitutively express NICD. Cells in such ectopic structures express biliary

markers and display apical ciliogenesis (Jeliazkova et al., 2013; Tchorz et al., 2009; Zong et al., 2009). Together, these data demonstrate that Notch signaling also controls biliary morphogenesis and induces biliary tubulogenesis.

Since activation of Notch signaling in a Notch receptor-expressing cell requires direct interaction with a cell harboring ligands at its surface, Notch ligand-expressing cells are expected to be located near hepatoblasts committed to differentiate into cholangiocytes. *Jag1* is expressed in portal endothelium, portal mesenchyme as well as in biliary cells, while Notch1 and Notch2 are expressed at the surface of biliary cells (Flynn et al., 2004; Kodama et al., 2004; McCright et al., 2002; Tanimizu and Miyajima, 2004; Zong et al., 2009). In mice, conditional deletion of *Jag1* using cell-specific Cre-recombinase shows that *Jag1* expression is not required in hepatoblasts and portal endothelium for bile duct development, while its absence in portal mesenchyme leads to bile duct paucity (Hofmann et al., 2010; Loomes et al., 2007). In cholangiocyte precursors, only Notch2 is necessary for bile duct morphogenesis (Geisler et al., 2008). In cultured hepatoblasts, biliary fate commitment and ductular morphogenesis induced by Sall4 overexpression are coupled with Notch2 and Jagged1 up-regulation (Oikawa et al., 2009) (Fig. 9).

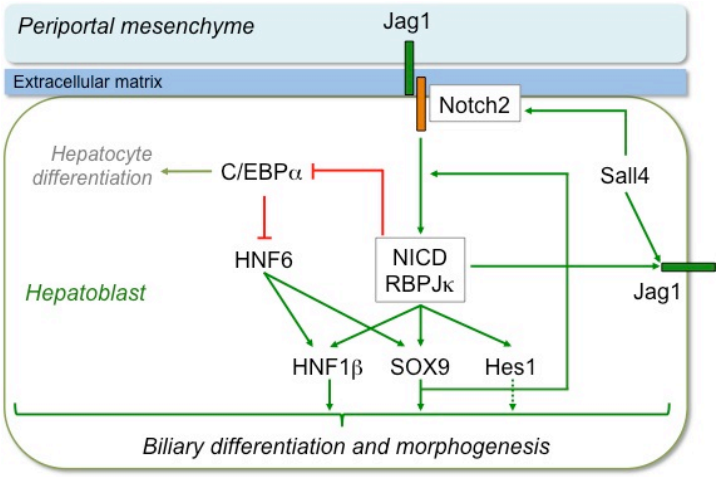

Figure 9: Schematic overview of Notch signaling pathway controlling bile duct development.

These data underline the role of Jagged1 in the portal mesenchyme, but suggest an involvement of Jagged1 in the cholangiocytes and/or hepatoblasts. Indeed, Jagged1 is expressed first on the portal side of PDS and then in all cholangiocytes lining symmetrical bile ducts, while Hairy and Enhancer of Split-1 (Hes1) – a transcription factor and target of Notch signaling – is already expressed in

hepatoblasts lining the parenchymal side of PDS before they start expressing biliary markers. Thus, the dynamic profile of Jagged1 and Hes1 in biliary precursors is consistent with a mechanism whereby radial differentiation of the PDS cells to symmetrical ducts lined by cholangiocytes depends on cell-cell interactions mediated by Jagged and Notch (Zong et al., 2009).

How Jagged1-induced Notch activation promotes biliary development is not well understood. Hes1-null mice do not form bile ducts (Kodama et al., 2004). Thus, Notch signaling could act on bile duct development via induction of Hes1. However, hepatoblast-specific deletion of Hes1 does not show a biliary phenotype and does not block ectopic biliary differentiation and tubulogenesis in livers of transgenic mice for NICD (Jeliazkova et al., 2013). Therefore, conflicting results persist and a clear role of Hes1 in biliary development has not been uncovered. On the other hand, activation of Notch signaling in cultured hepatoblasts induces HNF1β expression and represses C/EBPα, two transcription factors which positively and negatively regulate bile duct development, respectively (Tanimizu and Miyajima, 2004). Consistently, expression of HNF1β and SOX9 is strongly increased in hepatoblasts of transgenic mice overexpressing NICD (Jeliazkova et al., 2013; Tchorz et al., 2009; Zong et al., 2009). Notch signaling may target Sox9 directly: RBPJκ consensus sites are located upstream of the *Sox9* promoter region and are bound by NICD in chromatin immunoprecipitation (ChIP) experiments (Zong et al., 2009). In zebrafish larvae, Notch signaling also regulates expression of Sox9b during bile duct development. Reciprocally, Sox9b is also required to maintain Notch signaling in biliary cells. Therefore, Notch and Sox9b interact in positive feedback loop to ensure the development of zebrafish biliary system (Delous et al., 2012).

HNF6 mRNA expression is unaffected upon *in vivo* and *in vitro* activation of Notch signaling (Tanimizu and Miyajima, 2004; Zong et al., 2009). These data suggest that Notch signaling does not directly regulate HNF6 expression. However, double liver-specific deletion of HNF6 and RBPJκ results in biliary anomalies associated with loss of HNF1β and SOX9 expression, while single deletion of HNF6 or RBPJκ only marginally affects biliary development (Vanderpool et al., 2012). These data suggest that HNF6 and Notch cooperate by regulating HNF1β and SOX9 (Fig. 9).

1.3.4.3. Hippo/Yap signaling pathway in bile duct development

The Hippo/Yap signaling pathway is an important regulator of cell proliferation and organ size. However, recent studies underline its role in the control of cell fate and, notably in regulation of hepatobiliary cell fate decision. Hippo pathway consists of protein kinases which form a cascade leading to Yes-associated protein (Yap) phosphorylation and to its cytoplasmic sequestration and degradation. Inactivation of the Hippo pathway results in nuclear translocation of Yap where it activates transcription of target genes mostly by interacting with TEA domain transcription factors (TEAD1-4). Merlin/Neurofibromin 2 (Nf2) is located upstream of the Hippo pathway and regulates its activity in mammals (Zhang et al., 2008; Zhao et al., 2010; Zhao et al., 2007).

Yap plays a critical role in biliary development. Cholangiocytes express high levels of nuclear Yap, while Yap is preferentially detected in the cytoplasm of hepatocytes. Moreover, Yap target genes are enriched in purified cholangiocytes as compared to hepatocytes (Yimlamai et al., 2014). Liver-specific deletion of *Yap* reduces the number of ductal plate cells and bile ducts fail to develop. Consistently, *Nf2*-deleted hepatoblasts show strongly increased Yap activity resulting in biliary hyperplasia: at the end of gestation, the number of biliary cells is increased and the ductal plate has entirely developed into bile ducts. In adult mice, liver lobes are scattered with duct-like structures surrounded by stroma (Zhang et al., 2010). Therefore, Yap induces biliary fate commitment and stimulates bile duct morphogenesis. This is further supported by the observation that transgenic overexpression of TEAD2-dominant negative protein or administration of a drug disrupting the TEAD-Yap complex reduces biliary hyperplasia in *Nf2*-deficient livers (Liu-Chittenden et al., 2012) (Fig. 10).

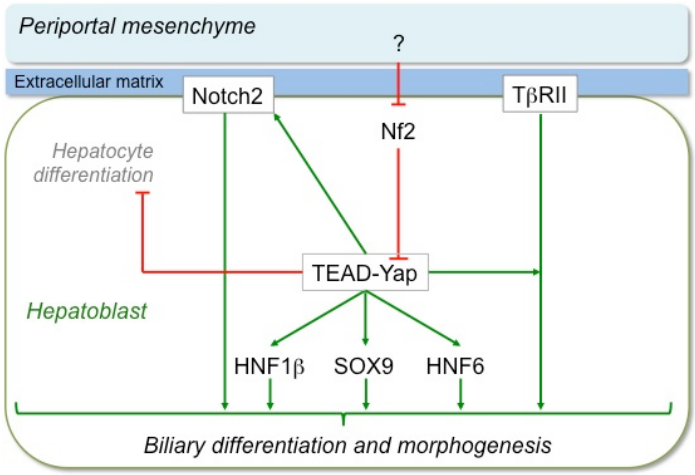

Figure 10: Schematic overview of the Hippo/Yap signaling pathway controlling bile duct development.

In addition, transgenic overexpression of Yap in adult hepatocytes induces their dedifferentiation. Hepatocytes adopt a biliary morphology and express biliary markers such as HNF1β, SOX9 and CK19, while HNF4 expression decreases and cells eventually form ductal structures surrounded by mesenchyme. Microarray analyses of these transdifferentiated hepatocytes reveal increased expression of genes associated with embryonic biliary development and ductal features as compared to control hepatocytes. TGFβ and Notch pathway signature genes, as well as SOX9, HNF1β and HNF6 factors belong to the up-regulated genes. Moreover, TEAD binding sites are found in SOX9 and Notch2 promoters. ChIP experiments confirm that SOX9 and Notch2 are direct targets of the TEAD-Yap complex in transdifferentiated hepatocytes. Finally, combined deletion of RBPJκ with transgenic overexpression of Yap partially rescues the phenotype (Yimlamai et al., 2014). Together, these data show that the TEAD-Yap complex controls bile duct development and imposes a biliary fate on adult hepatocytes, at least in part through Notch activation (Fig. 10).

1.3.4.4. Other signaling pathways in bile duct development

The study of canonical Wnt/β-catenin pathway in bile duct development faces a number of difficulties. In particular, the multitude of Wnt ligands and receptors makes this signaling pathway complex to study by using mutant mouse models. *Ex*

vivo experiments initially revealed that adding Wnt3a – a ligand of the canonical Wnt pathway – to cultured liver explants stimulates biliary differentiation (Hussain et al., 2004). Mutant mouse models further supported these conclusions. Mice with liver-specific deletion of *Adenomatosis Poliposis Coli* (*APC*) stabilize β-catenin in hepatoblasts. Such stabilization promotes development of numerous duct-like structures at the expense of hepatocyte differentiation and maturation (Decaens et al., 2008). Reciprocally, Tan *et al.* shows that deletion of β-catenin inhibited biliary differentiation leading to bile duct paucity (Tan et al., 2008). Together, these data suggest that Wnt/β-catenin signaling stimulates bile duct development. However, our laboratory has recently re-investigated the phenotype of β-catenin-deleted mice. These mutant mice indeed show strong defects in hepatocyte maturation and liver architecture, but differentiation of cholangiocytes was detectable (Beaudry et al., unpublished data). At later stages bile duct formation is impaired, most likely as a result from the deficient tissular architecture. Thus, Wnt/β-catenin is critical for hepatocyte maturation but not for biliary differentiation (Fig. 11). However, the β-catenin-independent non-canonical Wnt pathway seems to inhibit the formation of bile ducts. Indeed, liver-deletion of Wnt5a – a ligand of non-canonical Wnt pathway – promotes bile duct formation while adding Wnt5a inhibits duct-like structure morphogenesis of hepatoblasts when cultured in collagen gel (Kiyohashi et al., 2013).

FGF signaling is essential at the onset of liver development (see § 1.2.1 Development of hepatoblasts from the endoderm), but also plays a role in bile duct differentiation. In chick embryos, FGF receptors 1 and 2 (FGFR-1, -2) are specifically expressed in developing bile ducts. Moreover, high levels of the biliary markers Hhex and CK19 are strongly detected in chick liver explants when culture medium is complemented with FGF2 or FGF7 (Yanai et al., 2008). This suggests that FGF signaling promotes hepatoblast differentiation into cholangiocytes. FGF signaling synergizes with BMP4 and ECM components during biliary fate commitment. Indeed, BMP4, laminin and fibronectin enhance FGF-induced cholangiocyte differentiation (Yanai et al., 2008). α1- and α5-containing laminins are found in ECM surrounding developing bile ducts. α1-containing laminin, produced by periportal fibroblasts, is detected around PDS, and decreases during development to become undetectable after birth. α-5 containing laminin displays an opposite profile: it is produced by biliary precursors, and is first expressed along the portal side of PDS. Later it surrounds mature bile ducts and this persists in adult mice (Tanimizu et al., 2012). In three-dimensional cultures laminin α1 promotes apico-basal polarization of hepatoblasts and formation of cyst-like

structures (Tanimizu et al., 2007). Hepatoblast polarization and lumen formation are impaired when β1 integrin - a receptor of laminin - is blocked. Consistent with its expression profile, laminin α-5 is neither necessary for initiation of duct morphogenesis nor for cholangiocyte polarization but is essential for duct maturation and maintenance (Tanimizu et al., 2012). To summarize, laminin α1 is essential for biliary commitment, apico-basal polarization and PDS initiation whereas laminin α5 is necessary to develop mature bile ducts. This mechanism requires β1 integrin and is connected with FGF signaling (Fig. 11).

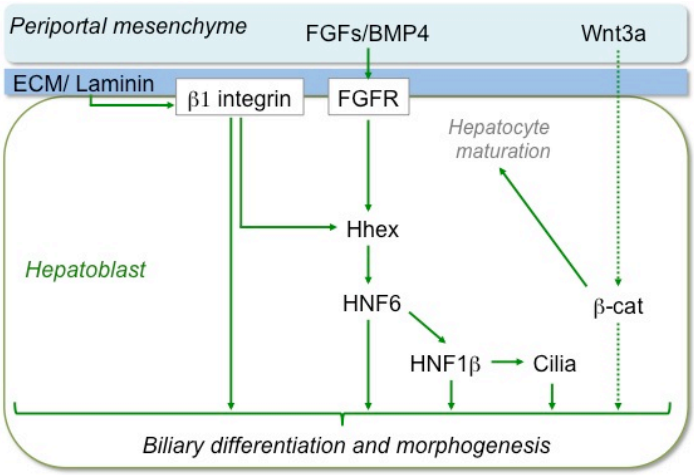

Figure 11: Schematic overview of the role of FGF, Wnt, laminins and ciliogenesis on bile duct development.

Primary cilia are found at the apical pole of cholangiocytes. They function as mechano-, chemo- and osmo-sensors that monitor and transmit luminal bile flow stimuli in biliary epithelia (Gradilone et al., 2007; Masyuk et al., 2008; Masyuk et al., 2006). Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD), which are characterized by progressive cyst development in kidney, liver, and pancreas, are cilia-related diseases. ADPKD and ARPKD are caused by mutations in *polycystin-1* (PC-1) or *polycystin-2* (PC-2), and *polycystic kidney and hepatic disease 1* (PKHD1), respectively; the three corresponding proteins are localized to primary cilia (Masyuk et al., 2003; Pazour et al., 2002; Yoder et al., 2002). Cyst formation in polycystic liver disease is considered to result from cilia defect-dependent overproliferation (Masyuk and LaRusso, 2006b; Strazzabosco and Somlo, 2011). However, recent data from our laboratory provide evidence that cyst formation in

the prenatal period is mainly due to an increased and accelerated differentiation of hepatoblasts into cholangiocyte precursors, while proliferation only promotes cyst expansion at a postnatal stage (Beaudry *et al.*, unpublished data). Therefore, the fact that cilia can modulate several signaling pathways involved in biliary fate commitment should be investigated as a potential cause of ciliary-dependent polycystic disease initiation (Clement et al., 2013; Habbig et al., 2011; Leitch et al., 2014).

As mentioned above, heterozygous mutations of HNF1β in humans, as well as HNF1β-deletion in liver, can lead to paucity or absence of normal primary cilia and thus, may lead to ductal plate malformation and cholestasis, as observed in these patients. Therefore, HNF1β may regulate biliary development by impacting on cilia development (Raynaud et al., 2011a; Raynaud et al., 2011b; Roelandt et al., 2012) (Fig. 11).

In conclusion, TGFβ, Notch, Hippo/Yap and FGF signaling, as well as ciliogenesis and signals from ECM, tightly control cholangiocyte fate commitment and bile duct morphogenesis. These inputs converge on the transcription factor network regulating hepatocyte *versus* biliary commitment and bile duct morphogenesis.

1.4. SOX4 and SOX9, two members of the SOX family

SOX transcription factors play multiple roles during development. They can maintain the pluripotent state of stem cells or the undifferentiated state in a progenitor pool. Conversely, they govern cell fate decision and differentiation in several processes, and can induce proliferation and apoptosis. The transcriptional activity of SOX proteins is not restricted to developmental processes; they also control homeostasis in adult tissues and act as oncogene or tumor suppressor in tumorigenesis.

1.4.1. SOX protein structure and molecular properties

SOX factors have been discovered in the early 1990s with the identification of *Sry* (sex-determining region on the Y chromosome), the gene responsible for male differentiation located on the Y chromosome. *Sry* possesses a non-canonical high mobility group (HMG) domain, also called HMG box, which allows it to bind to DNA and exert the function of a transcription factor (Sinclair et al., 1990). Given the important role of *Sry* in development, an intensive search for others proteins sharing a high degree of similarity with Sry was performed. This unveiled the Sry-related HMG box (SOX) family (Denny et al., 1992; Gubbay et al., 1990; Wright et al., 1993).

SOX factors are found in all metazoans. Their number amounts to 20 in mice and humans, and are classified in 8 groups called SOX A to H (Fig. 12A). SOX proteins within the same group have a high degree of similarity (≥ 80%) in the HMG box domain and high similarity in other domains; SOX from different groups have lower level of similarity (≈ 50%) in the HMG box and no significant homology outside this domain (Lefebvre et al., 2007) (Fig. 12B). SOX factors belonging to the same group, are often functionally redundant when they are co-expressed, while such redundancy between SOX proteins from distinct groups has not been found.

The HMG box is a 79 amino acid-long domain which displays DNA-binding, DNA-bending, protein interacting and nuclear localization properties. Its twisted L-shaped conformation consisting of three α helices confers the ability to specifically bind and bend the DNA (Bernard and Harley, 2010) (Fig. 12C). SOX proteins do not recognize a strict consensus sequence but exhibit preference for the hexameric core sequence 5'-(A/T)(A/T)CAA(A/T)-3'. The flanking sequences – depending on

SOX factors - also play a role in the binding affinity. Like other transcription factor harboring a HMG domain, SOX factors interact with and widen the minor groove of the DNA, thereby unwinding the DNA. This conformational change in DNA should facilitate the recruitment of other transcription factors and could be essential for the assembly of enhanceosomes (Kamachi and Kondoh, 2013; Lefebvre et al., 2007). This is illustrated by the fact that a mutation in human Sry gene causing the unability to bend DNA without affecting DNA binding is associated with complete gonadal dysgenesis (Pontiggia et al., 1994). In addition, SOX proteins have the ability to interact with several types of transcription factors through their HMG box domain. It is now agreed that binding of a SOX factor alone to a gene regulatory region does not activate transcription, the factor needs to co-bind with a specific partner which binds to a nearby site of DNA in order to create a functionally active complex. The interaction between two transcription factors dramatically enhances the low sequence specificity and binding affinity of one SOX factor alone. The fact that SOX factors contact the minor groove of DNA whereas most other transcription factors contact the major groove of DNA enables the formation of SOX-partner complex (Bernard and Harley, 2010; Kamachi and Kondoh, 2013; Kondoh and Kamachi, 2010; Lefebvre et al., 2007).

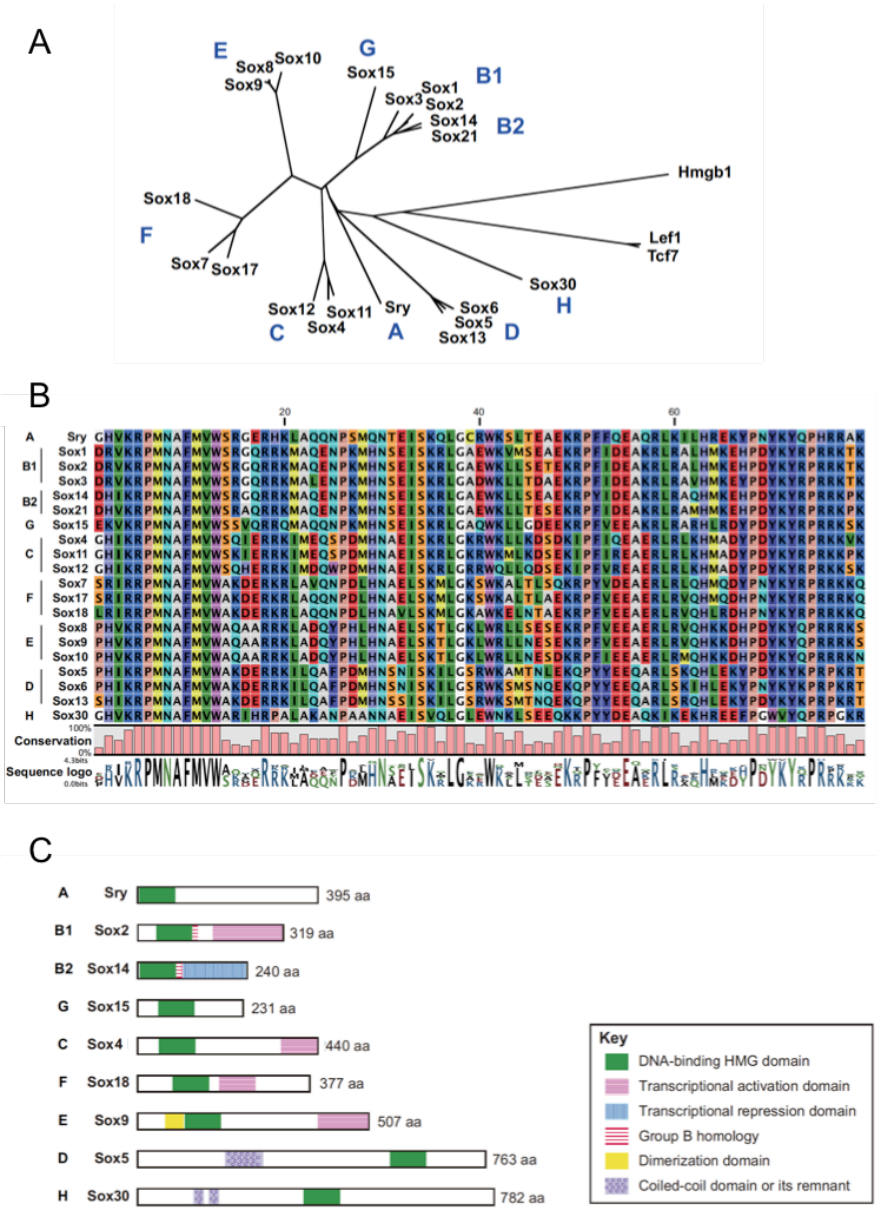

Figure 12: Phylogenetic tree and structure of SOX proteins (adapted from Kamachi and Kondoh, 2013). **A.** An unrooted phylogenetic neighbor-joining tree of the high mobility group (HMG) domains of the mouse in comparison with outgroup HMG domains Lef1/Tcf7 (lymphoid enhancer binding factor1/transcription factor 7, T cell specific) and Hmgb1. **B.** Alignment and classification of the SOX HMG domains. Sequences (79 amino acids) of the HMG domains from 20 mouse SOX proteins are aligned. The conservation level of the amino acid sequences are shown below the alignment with sequence logos. **C.** The domain structure of mouse SOX proteins, showing representatives of the groups. The basic structures are highly conserved among members in each group.

Most SOX factors have a transactivation domain (TAD) (SOX groups B1, C, E and F; some publications describe a TAD in the SOX A and H factors) or transrepression domain (TRD) (SOX B2, and potentially also SOX G proteins) in their C-terminal region (Fig. 12C). Therefore, these SOX factors contribute with their partner to the activity of enhanceosomes not only through their architectural role, but also through direct recruitment of co-activators or co-repressors. For example, it has been shown that the TAD of SOX9 directly interacts with Med12/Trap230 subunit of the RNA polymerase II mediator complex and that this interaction is essential for neural crest, cartilage and ear development (Rau et al., 2006; Zhou et al., 2002).

SOX D (SOX5/6/13) and SOX E (SOX8/9/10) also harbor a dimerization domain (referred to as coiled-coil domain in SOXD group). Homodimerization of these proteins increases their DNA-binding affinity to pairs of adjacent binding sites (Bernard et al., 2003; Sock et al., 2003) (Fig. 12C).

1.4.2. Developmental and tumorigenic functions of SOX4 and SOX9

SOX factors are involved in a multitude of developmental processes. In this paragraph, description of the developmental roles of SOX4 and SOX9 is focused on those which are relevant to the thesis, and is detailed in Table 1.

SOX4 and SOX9 are widely expressed during embryogenesis. SOX4-null mice die at E14 from circulatory failure due to strong defects in heart outflow tract formation, while SOX4 heterozygous mice survive but develop osteopenia (Nissen-Meyer et al., 2007; Schilham et al., 1996). SOX9-null mice die at E11-E12 presumably from congestive heart failure, since the major blood vessels are severely dilated. SOX9 heterozygous mice also die shortly after birth because of severe respiratory failure caused by a cleft secondary palate (Akiyama et al., 2004; Bi et al., 2001). Despite the numerous and essential roles of SOX4 during mouse embryogenesis, no mutation in its human orthologue has yet been associated with any congenital malformation (Penzo-Mendez, 2010). In contrast, heterozygous mutation in *Sox9* cause a severe skeletal malformation syndrome called Campomelic Dysplasia and is associated in about ¾ of XY individuals with sex-reversal (Foster et al., 1994; Wagner et al., 1994).

SOX4 and SOX9 contribute to determine tumor prognosis but their mode of action in tumorigenesis is sometimes controversial. SOX4 is highly expressed in

medulloblastomas, bladder, prostate, colon and non-small cell lung tumors, while SOX9 is associated with bladder, ovary, prostate, intestine and brain tumors (Penzo-Mendez, 2010; Pritchett et al., 2011). When considering liver cancer, high levels of SOX4 are found in hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) and up-regulation of SOX4 stabilizes and thus activates the oncogenic β-catenin; SOX4 also promotes tumor metastasis (Chen et al., 2013; Lahousse et al., 2011; Liao et al., 2008; Wang et al., 2014). In contrast, it has been shown that high SOX4 expression is correlated with reduced risk of recurrence and improved survival time in HCC patients (Hur et al., 2010). Regarding SOX9, high level of expression correlates with HCC progression and is predictive of poor prognosis (Guo et al., 2012; Zhang et al., 2012).

Table 1: Overview of the roles of SOX4 and SOX9 in development

	Roles	**References**
SOX4	**Cardiogenesis**: outflow tract formation; regulates differentiation and cytoskeletal organization of cardial neural crest cells and mesodermal cells Target gene: Cx43; Partner: Tbx3	(Boogerd et al., 2011; Paul et al., 2014; Schilham et al., 1996)
	Lymphopoiesis: required for survival of pro-B cells and for T-cell differentiation Partner: Syntenin	(Geijsen et al., 2001; Schilham et al., 1997; Sun et al., 2013)
	Pancreas development: required for normal development of pancreatic islets	(Mavropoulos et al., 2005; Wilson et al., 2005)
	Central nervous system: required for differentiation of thyrotrope and gonadotrope cells in the pituitary gland Target gene: Gata2	(Quiroz et al., 2012)
	Central nervous system: required for cell survival of neural cell types during spinal cord development	(Thein et al., 2010)

	Central nervous system: required for cell survival of neural progenitors Target gene: Tead2; Partner: Tead2	(Bhattaram et al., 2010; Potzner et al., 2010)
	Central nervous system: required for pan-neuronal protein expression, maturation of neurons and glial cells Target gene: Tubb3	(Bergsland et al., 2006; Cizelsky et al., 2013; Jiang et al., 2013; Usui et al., 2013)
	Eye development: required for survival of retinal ganglion cells	(Cizelsky et al., 2013; Jiang et al., 2013; Usui et al., 2013)
	Renal development: required for podocyte differentiation and nephron formation	(Huang et al., 2013)
	Ossification: required for osteoblast differentiation and proliferation	(Barrionuevo and Scherer, 2010; Nissen-Meyer et al., 2007; Sekido and Lovell-Badge, 2008)
	Skeletal muscle development: required for myoblasts differentiation Target gene: Caldesmon	(Jang et al., 2013)
SOX9	**Sex determination**: required for Sertoli cell development and inhibition of female differentiation Target gene: AMH; Partner: SF-1	(Barrionuevo and Scherer, 2010; Sekido and Lovell-Badge, 2008)
	Chondrogenesis: required for chondrocyte specification and early differentiation Target genes: Col2a1, Col9a1, Col11a2, Col27a1, Aggregan; Partners: SOX5/SOX6 and SOX9	(Lefebvre et al., 2007; Liu et al., 2007)

	Pancreas development: required for progenitor cell pool maintenance, differentiation of endocrine cells and formation of intrapancreatic duct Target gene: Ngn3 (endocrine differentiation)	(Delous et al., 2012; Lynn et al., 2007; Manfroid et al., 2012; Seymour et al., 2007)
	Gut development: required for differentiation of Paneth cells	(Mori-Akiyama et al., 2007)
	Cardiogenesis: required for endocardial cushion formation and cardiac valves development	(Akiyama et al., 2004; Lincoln et al., 2007)
	Hair follicle: required for specification of hair follicles stem cells and differentiation of skin epithelial cell lineages	(Vidal et al., 2005)
	Biliary development: required for intrahepatic and extrahepatic bile duct and for gallbladder development.	(Antoniou et al., 2009; Delous et al., 2012; Manfroid et al., 2012)
	Ear formation: required for invagination of otic placode	(Barrionuevo et al., 2008)
	Prostate development: required for the early differentiation of the prostate bud epithelia	(Thomsen et al., 2008)
	Neural crest: required for survival of neural stem cells, specification of the glial lineage and for cell migration through induction of epithelial-mesenchymal transition Target gene: Snail2; Partner: Snail2	(Haldin and LaBonne, 2010; Sakai et al., 2006; Scott et al., 2010)

1.5. References

Akai, Y., Oitate, T., Koike, T., Shiojiri, N., 2014. Impaired hepatocyte maturation, abnormal expression of biliary transcription factors and liver fibrosis in C/EBPalpha(Cebpa)-knockout mice. Histology and histopathology 29, 107-125.

Akiyama, H., Chaboissier, M.C., Behringer, R.R., Rowitch, D.H., Schedl, A., Epstein, J.A., de Crombrugghe, B., 2004. Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. Proceedings of the National Academy of Sciences of the United States of America 101, 6502-6507.

Alagille, D., Estrada, A., Hadchouel, M., Gautier, M., Odievre, M., Dommergues, J.P., 1987. Syndromic paucity of interlobular bile ducts (Alagille syndrome or arteriohepatic dysplasia): review of 80 cases. The Journal of pediatrics 110, 195-200.

Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., Lemaigre, F.P., 2009. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325-2333.

Arriazu, E., Ruiz de Galarreta, M., Cubero, F.J., Varela-Rey, M., Perez de Obanos, M.P., Leung, T.M., Lopategi, A., Benedicto, A., Abraham-Enachescu, I., Nieto, N., 2014. Extracellular matrix and liver disease. Antioxidants & redox signaling 21, 1078-1097.

Barrionuevo, F., Naumann, A., Bagheri-Fam, S., Speth, V., Taketo, M.M., Scherer, G., Neubuser, A., 2008. Sox9 is required for invagination of the otic placode in mice. Developmental biology 317, 213-224.

Barrionuevo, F., Scherer, G., 2010. SOX E genes: SOX9 and SOX8 in mammalian testis development. The international journal of biochemistry & cell biology 42, 433-436.

Bataller, R., Brenner, D.A., 2005. Liver fibrosis. The Journal of clinical investigation 115, 209-218.

Battle, M.A., Konopka, G., Parviz, F., Gaggl, A.L., Yang, C., Sladek, F.M., Duncan, S.A., 2006. Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. Proceedings of the National Academy of Sciences of the United States of America 103, 8419-8424.

Benhamouche, S., Decaens, T., Godard, C., Chambrey, R., Rickman, D.S., Moinard, C., Vasseur-Cognet, M., Kuo, C.J., Kahn, A., Perret, C., Colnot, S., 2006. Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. Developmental cell 10, 759-770.

Berg, T., Rountree, C.B., Lee, L., Estrada, J., Sala, F.G., Choe, A., Veltmaat, J.M., De Langhe, S., Lee, R., Tsukamoto, H., Crooks, G.M., Bellusci, S., Wang, K.S., 2007. Fibroblast growth factor 10 is critical for liver growth during embryogenesis

and controls hepatoblast survival via beta-catenin activation. Hepatology 46, 1187-1197.

Bergsland, M., Werme, M., Malewicz, M., Perlmann, T., Muhr, J., 2006. The establishment of neuronal properties is controlled by Sox4 and Sox11. Genes & development 20, 3475-3486.

Bernard, P., Harley, V.R., 2010. Acquisition of SOX transcription factor specificity through protein-protein interaction, modulation of Wnt signalling and post-translational modification. The international journal of biochemistry & cell biology 42, 400-410.

Bernard, P., Tang, P., Liu, S., Dewing, P., Harley, V.R., Vilain, E., 2003. Dimerization of SOX9 is required for chondrogenesis, but not for sex determination. Human molecular genetics 12, 1755-1765.

Bhattaram, P., Penzo-Mendez, A., Sock, E., Colmenares, C., Kaneko, K.J., Vassilev, A., Depamphilis, M.L., Wegner, M., Lefebvre, V., 2010. Organogenesis relies on SoxC transcription factors for the survival of neural and mesenchymal progenitors. Nature communications 1, 9.

Bi, W., Huang, W., Whitworth, D.J., Deng, J.M., Zhang, Z., Behringer, R.R., de Crombrugghe, B., 2001. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. Proceedings of the National Academy of Sciences of the United States of America 98, 6698-6703.

Blouin, A., Bolender, R.P., Weibel, E.R., 1977. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. The Journal of cell biology 72, 441-455.

Boogerd, C.J., Wong, L.Y., van den Boogaard, M., Bakker, M.L., Tessadori, F., Bakkers, J., t Hoen, P.A., Moorman, A.F., Christoffels, V.M., Barnett, P., 2011. Sox4 mediates Tbx3 transcriptional regulation of the gap junction protein Cx43. Cellular and molecular life sciences : CMLS 68, 3949-3961.

Bort, R., Signore, M., Tremblay, K., Martinez Barbera, J.P., Zaret, K.S., 2006. Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. Developmental biology 290, 44-56.

Bossard, P., Zaret, K.S., 1998. GATA transcription factors as potentiators of gut endoderm differentiation. Development 125, 4909-4917.

Calmont, A., Wandzioch, E., Tremblay, K.D., Minowada, G., Kaestner, K.H., Martin, G.R., Zaret, K.S., 2006. An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. Developmental cell 11, 339-348.

Carpentier, R., Suner, R.E., van Hul, N., Kopp, J.L., Beaudry, J.B., Cordi, S., Antoniou, A., Raynaud, P., Lepreux, S., Jacquemin, P., Leclercq, I.A., Sander, M., Lemaigre, F.P., 2011. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 141, 1432-1438, 1438 e1431-1434.

Chen, X., Zhang, L., Zhang, T., Hao, M., Zhang, X., Zhang, J., Xie, Q., Wang, Y., Guo, M., Zhuang, H., Lu, F., 2013. Methylation-mediated repression of microRNA 129-2 enhances oncogenic SOX4 expression in HCC. Liver international : official journal of the International Association for the Study of the Liver 33, 476-486.

Cheung, I.D., Bagnat, M., Ma, T.P., Datta, A., Evason, K., Moore, J.C., Lawson, N.D., Mostov, K.E., Moens, C.B., Stainier, D.Y., 2012. Regulation of intrahepatic biliary duct morphogenesis by Claudin 15-like b. Developmental biology 361, 68-78.

Cirillo, L.A., Lin, F.R., Cuesta, I., Friedman, D., Jarnik, M., Zaret, K.S., 2002. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Molecular cell 9, 279-289.

Cizelsky, W., Hempel, A., Metzig, M., Tao, S., Hollemann, T., Kuhl, M., Kuhl, S.J., 2013. sox4 and sox11 function during Xenopus laevis eye development. PloS one 8, e69372.

Clement, C.A., Ajbro, K.D., Koefoed, K., Vestergaard, M.L., Veland, I.R., Henriques de Jesus, M.P., Pedersen, L.B., Benmerah, A., Andersen, C.Y., Larsen, L.A., Christensen, S.T., 2013. TGF-beta signaling is associated with endocytosis at the pocket region of the primary cilium. Cell reports 3, 1806-1814.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., Lemaigre, F.P., 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes & development 19, 1849-1854.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129, 1819-1828.

Clotman, F., Libbrecht, L., Gresh, L., Yaniv, M., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2003. Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development. J Hepatol 39, 686-692.

Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., Barra, J., 2002. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129, 1829-1838.

Decaens, T., Godard, C., de Reynies, A., Rickman, D.S., Tronche, F., Couty, J.P., Perret, C., Colnot, S., 2008. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology 47, 247-258.

Delous, M., Yin, C., Shin, D., Ninov, N., Debrito Carten, J., Pan, L., Ma, T.P., Farber, S.A., Moens, C.B., Stainier, D.Y., 2012. Sox9b is a key regulator of pancreaticobiliary ductal system development. PLoS genetics 8, e1002754.

Denny, P., Swift, S., Brand, N., Dabhade, N., Barton, P., Ashworth, A., 1992. A conserved family of genes related to the testis determining gene, SRY. Nucleic acids research 20, 2887.

Desmet, V., Roskams, T., Van Eyken, P., 1995. Ductular reaction in the liver. Pathology, research and practice 191, 513-524.

Emerick, K.M., Rand, E.B., Goldmuntz, E., Krantz, I.D., Spinner, N.B., Piccoli, D.A., 1999. Features of Alagille syndrome in 92 patients: frequency and relation to prognosis. Hepatology 29, 822-829.

Enomoto, H., Yoshida, K., Kishima, Y., Kinoshita, T., Yamamoto, M., Everett, A.D., Miyajima, A., Nakamura, H., 2002. Hepatoma-derived growth factor is highly expressed in developing liver and promotes fetal hepatocyte proliferation. Hepatology 36, 1519-1527.

Flynn, D.M., Nijjar, S., Hubscher, S.G., de Goyet Jde, V., Kelly, D.A., Strain, A.J., Crosby, H.A., 2004. The role of Notch receptor expression in bile duct development and disease. The Journal of pathology 204, 55-64.

Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kwok, C., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N., et al., 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372, 525-530.

Fu, D., Wakabayashi, Y., Ido, Y., Lippincott-Schwartz, J., Arias, I.M., 2010. Regulation of bile canalicular network formation and maintenance by AMP-activated protein kinase and LKB1. Journal of cell science 123, 3294-3302.

Fu, D., Wakabayashi, Y., Lippincott-Schwartz, J., Arias, I.M., 2011. Bile acid stimulates hepatocyte polarization through a cAMP-Epac-MEK-LKB1-AMPK pathway. Proceedings of the National Academy of Sciences of the United States of America 108, 1403-1408.

Geijsen, N., Uings, I.J., Pals, C., Armstrong, J., McKinnon, M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., Coffer, P.J., 2001. Cytokine-specific transcriptional regulation through an IL-5Ralpha interacting protein. Science 293, 1136-1138.

Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimber-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., Siveke, J.T., 2008. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48, 607-616.

Germain, L., Blouin, M.J., Marceau, N., 1988. Biliary epithelial and hepatocytic cell lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, alpha-fetoprotein, albumin, and cell surface-exposed components. Cancer research 48, 4909-4918.

Gissen, P., Johnson, C.A., Morgan, N.V., Stapelbroek, J.M., Forshew, T., Cooper, W.N., McKiernan, P.J., Klomp, L.W., Morris, A.A., Wraith, J.E., McClean, P., Lynch, S.A., Thompson, R.J., Lo, B., Quarrell, O.W., Di Rocco, M., Trembath, R.C., Mandel, H., Wali, S., Karet, F.E., Knisely, A.S., Houwen, R.H., Kelly, D.A., Maher, E.R., 2004. Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome. Nature genetics 36, 400-404.

Gradilone, S.A., Masyuk, A.I., Splinter, P.L., Banales, J.M., Huang, B.Q., Tietz, P.S., Masyuk, T.V., Larusso, N.F., 2007. Cholangiocyte cilia express TRPV4 and detect changes in luminal tonicity inducing bicarbonate secretion. Proceedings of the National Academy of Sciences of the United States of America 104, 19138-19143.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., Zaret, K.S., 1996. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes & development 10, 1670-1682.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 346, 245-250.

Guo, X., Xiong, L., Sun, T., Peng, R., Zou, L., Zhu, H., Zhang, J., Li, H., Zhao, J., 2012. Expression features of SOX9 associate with tumor progression and poor prognosis of hepatocellular carcinoma. Diagnostic pathology 7, 44.

Habbig, S., Bartram, M.P., Muller, R.U., Schwarz, R., Andriopoulos, N., Chen, S., Sagmuller, J.G., Hoehne, M., Burst, V., Liebau, M.C., Reinhardt, H.C., Benzing, T., Schermer, B., 2011. NPHP4, a cilia-associated protein, negatively regulates the Hippo pathway. The Journal of cell biology 193, 633-642.

Hadj-Rabia, S., Baala, L., Vabres, P., Hamel-Teillac, D., Jacquemin, E., Fabre, M., Lyonnet, S., De Prost, Y., Munnich, A., Hadchouel, M., Smahi, A., 2004. Claudin-1 gene mutations in neonatal sclerosing cholangitis associated with ichthyosis: a tight junction disease. Gastroenterology 127, 1386-1390.

Haldin, C.E., LaBonne, C., 2010. SoxE factors as multifunctional neural crest regulatory factors. The international journal of biochemistry & cell biology 42, 441-444.

Hand, N.J., Master, Z.R., Eauclaire, S.F., Weinblatt, D.E., Matthews, R.P., Friedman, J.R., 2009. The microRNA-30 family is required for vertebrate hepatobiliary development. Gastroenterology 136, 1081-1090.

Hofmann, J.J., Zovein, A.C., Koh, H., Radtke, F., Weinmaster, G., Iruela-Arispe, M.L., 2010. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 137, 4061-4072.

Homolya, L., Fu, D., Sengupta, P., Jarnik, M., Gillet, J.P., Vitale-Cross, L., Gutkind, J.S., Lippincott-Schwartz, J., Arias, I.M., 2014. LKB1/AMPK and PKA control ABCB11 trafficking and polarization in hepatocytes. PloS one 9, e91921.

Huang, J., Arsenault, M., Kann, M., Lopez-Mendez, C., Saleh, M., Wadowska, D., Taglienti, M., Ho, J., Miao, Y., Sims, D., Spears, J., Lopez, A., Wright, G., Hartwig, S., 2013. The transcription factor Sry-related HMG box-4 (SOX4) is required for normal renal development in vivo. Developmental dynamics : an official publication of the American Association of Anatomists 242, 790-799.

Hunter, M.P., Wilson, C.M., Jiang, X., Cong, R., Vasavada, H., Kaestner, K.H., Bogue, C.W., 2007. The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. Developmental biology 308, 355-367.

Hur, W., Rhim, H., Jung, C.K., Kim, J.D., Bae, S.H., Jang, J.W., Yang, J.M., Oh, S.T., Kim, D.G., Wang, H.J., Lee, S.B., Yoon, S.K., 2010. SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma: clinical implication and functional analysis in vitro. Carcinogenesis 31, 1298-1307.

Hussain, S.Z., Sneddon, T., Tan, X., Micsenyi, A., Michalopoulos, G.K., Monga, S.P., 2004. Wnt impacts growth and differentiation in ex vivo liver development. Experimental cell research 292, 157-169.

Jang, S.M., Kim, J.W., Kim, D., Kim, C.H., An, J.H., Choi, K.H., Rhee, S., 2013. Sox4-mediated caldesmon expression facilitates differentiation of skeletal myoblasts. Journal of cell science 126, 5178-5188.

Jeliazkova, P., Jors, S., Lee, M., Zimber-Strobl, U., Ferrer, J., Schmid, R.M., Siveke, J.T., Geisler, F., 2013. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology 57, 2469-2479.

Jiang, Y., Ding, Q., Xie, X., Libby, R.T., Lefebvre, V., Gan, L., 2013. Transcription factors SOX4 and SOX11 function redundantly to regulate the development of mouse retinal ganglion cells. The Journal of biological chemistry 288, 18429-18438.

Jung, J., Zheng, M., Goldfarb, M., Zaret, K.S., 1999. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 284, 1998-2003.

Kamachi, Y., Kondoh, H., 2013. Sox proteins: regulators of cell fate specification and differentiation. Development 140, 4129-4144.

Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T., Miyajima, A., 1999. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. The EMBO journal 18, 2127-2136.

Kiyohashi, K., Kakinuma, S., Kamiya, A., Sakamoto, N., Nitta, S., Yamanaka, H., Yoshino, K., Fujiki, J., Murakawa, M., Kusano-Kitazume, A., Shimizu, H., Okamoto, R., Azuma, S., Nakagawa, M., Asahina, Y., Tanimizu, N., Kikuchi, A., Nakauchi, H., Watanabe, M., 2013. Wnt5a signaling mediates biliary differentiation of fetal hepatic stem/progenitor cells in mice. Hepatology 57, 2502-2513.

Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., Chiba, T., 2004. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127, 1775-1786.

Kondoh, H., Kamachi, Y., 2010. SOX-partner code for cell specification: Regulatory target selection and underlying molecular mechanisms. The international journal of biochemistry & cell biology 42, 391-399.

Krupczak-Hollis, K., Wang, X., Kalinichenko, V.V., Gusarova, G.A., Wang, I.C., Dennewitz, M.B., Yoder, H.M., Kiyokawa, H., Kaestner, K.H., Costa, R.H., 2004. The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. Developmental biology 276, 74-88.

Kyrmizi, I., Hatzis, P., Katrakili, N., Tronche, F., Gonzalez, F.J., Talianidis, I., 2006. Plasticity and expanding complexity of the hepatic transcription factor network during liver development. Genes & development 20, 2293-2305.

Lahousse, S.A., Hoenerhoff, M., Collins, J., Ton, T.V., Masinde, T., Olson, D., Rebolloso, Y., Koujitani, T., Tomer, K.B., Hong, H.H., Bucher, J., Sills, R.C., 2011. Gene expression and mutation assessment provide clues of genetic and epigenetic mechanisms in liver tumors of oxazepam-exposed mice. Veterinary pathology 48, 875-884.

Laudadio, I., Manfroid, I., Achouri, Y., Schmidt, D., Wilson, M.D., Cordi, S., Thorrez, L., Knoops, L., Jacquemin, P., Schuit, F., Pierreux, C.E., Odom, D.T., Peers, B., Lemaigre, F.P., 2012. A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. Gastroenterology 142, 119-129.

Lee, C.S., Friedman, J.R., Fulmer, J.T., Kaestner, K.H., 2005. The initiation of liver development is dependent on Foxa transcription factors. Nature 435, 944-947.

Lefebvre, V., Dumitriu, B., Penzo-Mendez, A., Han, Y., Pallavi, B., 2007. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. The international journal of biochemistry & cell biology 39, 2195-2214.

Leitch, C.C., Lodh, S., Prieto-Echague, V., Badano, J.L., Zaghloul, N.A., 2014. Basal body proteins regulate Notch signaling through endosomal trafficking. Journal of cell science 127, 2407-2419.

Lemaigre, F.P., 2009. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 137, 62-79.

Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., Costa, T., Pierpont, M.E., Rand, E.B., Piccoli, D.A., Hood, L., Spinner, N.B., 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nature genetics 16, 243-251.

Liao, Y.L., Sun, Y.M., Chau, G.Y., Chau, Y.P., Lai, T.C., Wang, J.L., Horng, J.T., Hsiao, M., Tsou, A.P., 2008. Identification of SOX4 target genes using phylogenetic footprinting-based prediction from expression microarrays suggests that overexpression of SOX4 potentiates metastasis in hepatocellular carcinoma. Oncogene 27, 5578-5589.

Lincoln, J., Kist, R., Scherer, G., Yutzey, K.E., 2007. Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development. Developmental biology 305, 120-132.

Liu, C.J., Zhang, Y., Xu, K., Parsons, D., Alfonso, D., Di Cesare, P.E., 2007. Transcriptional activation of cartilage oligomeric matrix protein by Sox9, Sox5, and Sox6 transcription factors and CBP/p300 coactivators. Frontiers in bioscience : a journal and virtual library 12, 3899-3910.

Liu-Chittenden, Y., Huang, B., Shim, J.S., Chen, Q., Lee, S.J., Anders, R.A., Liu, J.O., Pan, D., 2012. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. Genes & development 26, 1300-1305.

Lokmane, L., Haumaitre, C., Garcia-Villalba, P., Anselme, I., Schneider-Maunoury, S., Cereghini, S., 2008. Crucial role of vHNF1 in vertebrate hepatic specification. Development 135, 2777-2786.

Loomes, K.M., Russo, P., Ryan, M., Nelson, A., Underkoffler, L., Glover, C., Fu, H., Gridley, T., Kaestner, K.H., Oakey, R.J., 2007. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 45, 323-330.

Lorent, K., Moore, J.C., Siekmann, A.F., Lawson, N., Pack, M., 2010. Reiterative use of the notch signal during zebrafish intrahepatic biliary development. Developmental dynamics : an official publication of the American Association of Anatomists 239, 855-864.

Lorent, K., Yeo, S.Y., Oda, T., Chandrasekharappa, S., Chitnis, A., Matthews, R.P., Pack, M., 2004. Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. Development 131, 5753-5766.

Ludtke, T.H., Christoffels, V.M., Petry, M., Kispert, A., 2009. Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. Hepatology 49, 969-978.

Lynn, F.C., Smith, S.B., Wilson, M.E., Yang, K.Y., Nekrep, N., German, M.S., 2007. Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 104, 10500-10505.

Manfroid, I., Ghaye, A., Naye, F., Detry, N., Palm, S., Pan, L., Ma, T.P., Huang, W., Rovira, M., Martial, J.A., Parsons, M.J., Moens, C.B., Voz, M.L., Peers, B., 2012. Zebrafish sox9b is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration. Developmental biology 366, 268-278.

Margagliotti, S., Clotman, F., Pierreux, C.E., Beaudry, J.B., Jacquemin, P., Rousseau, G.G., Lemaigre, F.P., 2007. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. Developmental biology 311, 579-589.

Masyuk, A.I., Gradilone, S.A., Banales, J.M., Huang, B.Q., Masyuk, T.V., Lee, S.O., Splinter, P.L., Stroope, A.J., Larusso, N.F., 2008. Cholangiocyte primary cilia are chemosensory organelles that detect biliary nucleotides via P2Y12 purinergic

receptors. American journal of physiology. Gastrointestinal and liver physiology 295, G725-734.

Masyuk, A.I., LaRusso, N.F., 2006a. Aquaporins in the hepatobiliary system. Hepatology 43, S75-81.

Masyuk, A.I., Masyuk, T.V., Splinter, P.L., Huang, B.Q., Stroope, A.J., LaRusso, N.F., 2006. Cholangiocyte cilia detect changes in luminal fluid flow and transmit them into intracellular Ca2+ and cAMP signaling. Gastroenterology 131, 911-920.

Masyuk, T., LaRusso, N., 2006b. Polycystic liver disease: new insights into disease pathogenesis. Hepatology 43, 906-908.

Masyuk, T.V., Huang, B.Q., Ward, C.J., Masyuk, A.I., Yuan, D., Splinter, P.L., Punyashthiti, R., Ritman, E.L., Torres, V.E., Harris, P.C., LaRusso, N.F., 2003. Defects in cholangiocyte fibrocystin expression and ciliary structure in the PCK rat. Gastroenterology 125, 1303-1310.

Matsumoto, K., Miki, R., Nakayama, M., Tatsumi, N., Yokouchi, Y., 2008. Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium. Developmental biology 319, 234-247.

Matthews, R.P., Plumb-Rudewiez, N., Lorent, K., Gissen, P., Johnson, C.A., Lemaigre, F., Pack, M., 2005. Zebrafish vps33b, an ortholog of the gene responsible for human arthrogryposis-renal dysfunction-cholestasis syndrome, regulates biliary development downstream of the onecut transcription factor hnf6. Development 132, 5295-5306.

Mavropoulos, A., Devos, N., Biemar, F., Zecchin, E., Argenton, F., Edlund, H., Motte, P., Martial, J.A., Peers, B., 2005. sox4b is a key player of pancreatic alpha cell differentiation in zebrafish. Developmental biology 285, 211-223.

McCright, B., Lozier, J., Gridley, T., 2002. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 129, 1075-1082.

McDaniell, R., Warthen, D.M., Sanchez-Lara, P.A., Pai, A., Krantz, I.D., Piccoli, D.A., Spinner, N.B., 2006. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. American journal of human genetics 79, 169-173.

McLin, V.A., Rankin, S.A., Zorn, A.M., 2007. Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development 134, 2207-2217.

Mori-Akiyama, Y., van den Born, M., van Es, J.H., Hamilton, S.R., Adams, H.P., Zhang, J., Clevers, H., de Crombrugghe, B., 2007. SOX9 is required for the differentiation of paneth cells in the intestinal epithelium. Gastroenterology 133, 539-546.

Nissen-Meyer, L.S., Jemtland, R., Gautvik, V.T., Pedersen, M.E., Paro, R., Fortunati, D., Pierroz, D.D., Stadelmann, V.A., Reppe, S., Reinholt, F.P., Del

Fattore, A., Rucci, N., Teti, A., Ferrari, S., Gautvik, K.M., 2007. Osteopenia, decreased bone formation and impaired osteoblast development in Sox4 heterozygous mice. Journal of cell science 120, 2785-2795.

Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S., Chandrasekharappa, S.C., 1997. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nature genetics 16, 235-242.

Oikawa, T., Kamiya, A., Kakinuma, S., Zeniya, M., Nishinakamura, R., Tajiri, H., Nakauchi, H., 2009. Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells. Gastroenterology 136, 1000-1011.

Parviz, F., Matullo, C., Garrison, W.D., Savatski, L., Adamson, J.W., Ning, G., Kaestner, K.H., Rossi, J.M., Zaret, K.S., Duncan, S.A., 2003. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nature genetics 34, 292-296.

Paul, M.H., Harvey, R.P., Wegner, M., Sock, E., 2014. Cardiac outflow tract development relies on the complex function of Sox4 and Sox11 in multiple cell types. Cellular and molecular life sciences : CMLS 71, 2931-2945.

Pazour, G.J., San Agustin, J.T., Follit, J.A., Rosenbaum, J.L., Witman, G.B., 2002. Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease. Current biology : CB 12, R378-380.

Penzo-Mendez, A.I., 2010. Critical roles for SoxC transcription factors in development and cancer. The international journal of biochemistry & cell biology 42, 425-428.

Plumb-Rudewiez, N., Clotman, F., Strick-Marchand, H., Pierreux, C.E., Weiss, M.C., Rousseau, G.G., Lemaigre, F.P., 2004. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. Hepatology 40, 1266-1274.

Pontiggia, A., Rimini, R., Harley, V.R., Goodfellow, P.N., Lovell-Badge, R., Bianchi, M.E., 1994. Sex-reversing mutations affect the architecture of SRY-DNA complexes. The EMBO journal 13, 6115-6124.

Potzner, M.R., Tsarovina, K., Binder, E., Penzo-Mendez, A., Lefebvre, V., Rohrer, H., Wegner, M., Sock, E., 2010. Sequential requirement of Sox4 and Sox11 during development of the sympathetic nervous system. Development 137, 775-784.

Pritchett, J., Athwal, V., Roberts, N., Hanley, N.A., Hanley, K.P., 2011. Understanding the role of SOX9 in acquired diseases: lessons from development. Trends in molecular medicine 17, 166-174.

Quiroz, Y., Lopez, M., Mavropoulos, A., Motte, P., Martial, J.A., Hammerschmidt, M., Muller, M., 2012. The HMG-box transcription factor Sox4b is required for pituitary expression of gata2a and specification of thyrotrope and gonadotrope cells in zebrafish. Molecular endocrinology 26, 1014-1027.

Rastegar, M., Rousseau, G.G., Lemaigre, F.P., 2000. CCAAT/enhancer-binding protein-alpha is a component of the growth hormone-regulated network of liver transcription factors. Endocrinology 141, 1686-1692.

Rau, M.J., Fischer, S., Neumann, C.J., 2006. Zebrafish Trap230/Med12 is required as a coactivator for Sox9-dependent neural crest, cartilage and ear development. Developmental biology 296, 83-93.

Raynaud, P., Carpentier, R., Antoniou, A., Lemaigre, F.P., 2011a. Biliary differentiation and bile duct morphogenesis in development and disease. The international journal of biochemistry & cell biology 43, 245-256.

Raynaud, P., Tate, J., Callens, C., Cordi, S., Vandersmissen, P., Carpentier, R., Sempoux, C., Devuyst, O., Pierreux, C.E., Courtoy, P., Dahan, K., Delbecque, K., Lepreux, S., Pontoglio, M., Guay-Woodford, L.M., Lemaigre, F.P., 2011b. A classification of ductal plate malformations based on distinct pathogenic mechanisms of biliary dysmorphogenesis. Hepatology 53, 1959-1966.

Roelandt, P., Antoniou, A., Libbrecht, L., Van Steenbergen, W., Laleman, W., Verslype, C., Van der Merwe, S., Nevens, F., De Vos, R., Fischer, E., Pontoglio, M., Lemaigre, F., Cassiman, D., 2012. HNF1B deficiency causes ciliary defects in human cholangiocytes. Hepatology 56, 1178-1181.

Rogler, C.E., Levoci, L., Ader, T., Massimi, A., Tchaikovskaya, T., Norel, R., Rogler, L.E., 2009. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. Hepatology 50, 575-584.

Roskams, T.A., Theise, N.D., Balabaud, C., Bhagat, G., Bhathal, P.S., Bioulac-Sage, P., Brunt, E.M., Crawford, J.M., Crosby, H.A., Desmet, V., Finegold, M.J., Geller, S.A., Gouw, A.S., Hytiroglou, P., Knisely, A.S., Kojiro, M., Lefkowitch, J.H., Nakanuma, Y., Olynyk, J.K., Park, Y.N., Portmann, B., Saxena, R., Scheuer, P.J., Strain, A.J., Thung, S.N., Wanless, I.R., West, A.B., 2004. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 39, 1739-1745.

Rossi, J.M., Dunn, N.R., Hogan, B.L., Zaret, K.S., 2001. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes & development 15, 1998-2009.

Sakai, D., Suzuki, T., Osumi, N., Wakamatsu, Y., 2006. Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development. Development 133, 1323-1333.

Schilham, M.W., Moerer, P., Cumano, A., Clevers, H.C., 1997. Sox-4 facilitates thymocyte differentiation. European journal of immunology 27, 1292-1295.

Schilham, M.W., Oosterwegel, M.A., Moerer, P., Ya, J., de Boer, P.A., van de Wetering, M., Verbeek, S., Lamers, W.H., Kruisbeek, A.M., Cumano, A., Clevers, H., 1996. Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. Nature 380, 711-714.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., Birchmeier, C., 1995. Scatter factor/hepatocyte growth factor is essential for liver development. Nature 373, 699-702.

Scott, C.E., Wynn, S.L., Sesay, A., Cruz, C., Cheung, M., Gomez Gaviro, M.V., Booth, S., Gao, B., Cheah, K.S., Lovell-Badge, R., Briscoe, J., 2010. SOX9 induces and maintains neural stem cells. Nature neuroscience 13, 1181-1189.

Sekido, R., Lovell-Badge, R., 2008. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 453, 930-934.

Seth, A., Ye, J., Yu, N., Guez, F., Bedford, D.C., Neale, G.A., Cordi, S., Brindle, P.K., Lemaigre, F.P., Kaestner, K.H., Sosa-Pineda, B., 2014. Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. Development 141, 538-547.

Seymour, P.A., Freude, K.K., Tran, M.N., Mayes, E.E., Jensen, J., Kist, R., Scherer, G., Sander, M., 2007. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 104, 1865-1870.

Shiojiri, N., 1984. The origin of intrahepatic bile duct cells in the mouse. Journal of embryology and experimental morphology 79, 25-39.

Shiojiri, N., 1997. Development and differentiation of bile ducts in the mammalian liver. Microscopy research and technique 39, 328-335.

Shiojiri, N., Koike, T., 1997. Differentiation of biliary epithelial cells from the mouse hepatic endodermal cells cultured in vitro. The Tohoku journal of experimental medicine 181, 1-8.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., Goodfellow, P.N., 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346, 240-244.

Slim, C.L., Lazaro-Dieguez, F., Bijlard, M., Toussaint, M.J., de Bruin, A., Du, Q., Musch, A., van Ijzendoorn, S.C., 2013. Par1b induces asymmetric inheritance of plasma membrane domains via LGN-dependent mitotic spindle orientation in proliferating hepatocytes. PLoS biology 11, e1001739.

Sock, E., Pagon, R.A., Keymolen, K., Lissens, W., Wegner, M., Scherer, G., 2003. Loss of DNA-dependent dimerization of the transcription factor SOX9 as a cause for campomelic dysplasia. Human molecular genetics 12, 1439-1447.

Spagnoli, F.M., Amicone, L., Tripodi, M., Weiss, M.C., 1998. Identification of a bipotential precursor cell in hepatic cell lines derived from transgenic mice expressing cyto-Met in the liver. The Journal of cell biology 143, 1101-1112.

Spence, J.R., Lange, A.W., Lin, S.C., Kaestner, K.H., Lowy, A.M., Kim, I., Whitsett, J.A., Wells, J.M., 2009. Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. Developmental cell 17, 62-74.

Stanulovic, V.S., Kyrmizi, I., Kruithof-de Julio, M., Hoogenkamp, M., Vermeulen, J.L., Ruijter, J.M., Talianidis, I., Hakvoort, T.B., Lamers, W.H., 2007. Hepatic HNF4alpha deficiency induces periportal expression of glutamine synthetase and other pericentral enzymes. Hepatology 45, 433-444.

Strazzabosco, M., Somlo, S., 2011. Polycystic liver diseases: congenital disorders of cholangiocyte signaling. Gastroenterology 140, 1855-1859, 1859 e1851.

Sun, B., Mallampati, S., Gong, Y., Wang, D., Lefebvre, V., Sun, X., 2013. Sox4 is required for the survival of pro-B cells. Journal of immunology 190, 2080-2089.

Suzuki, A., Sekiya, S., Buscher, D., Izpisua Belmonte, J.C., Taniguchi, H., 2008. Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development 135, 1589-1595.

Suzuki, T., Kanai, Y., Hara, T., Sasaki, J., Sasaki, T., Kohara, M., Maehama, T., Taya, C., Shitara, H., Yonekawa, H., Frohman, M.A., Yokozeki, T., Kanaho, Y., 2006. Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. Molecular and cellular biology 26, 6149-6156.

Takayama, K., Kawabata, K., Nagamoto, Y., Inamura, M., Ohashi, K., Okuno, H., Yamaguchi, T., Tashiro, K., Sakurai, F., Hayakawa, T., Okano, T., Furue, M.K., Mizuguchi, H., 2014. CCAAT/enhancer binding protein-mediated regulation of TGFbeta receptor 2 expression determines the hepatoblast fate decision. Development 141, 91-100.

Tan, X., Yuan, Y., Zeng, G., Apte, U., Thompson, M.D., Cieply, B., Stolz, D.B., Michalopoulos, G.K., Kaestner, K.H., Monga, S.P., 2008. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. Hepatology 47, 1667-1679.

Tanimizu, N., Kikkawa, Y., Mitaka, T., Miyajima, A., 2012. alpha1- and alpha5-containing laminins regulate the development of bile ducts via beta1 integrin signals. The Journal of biological chemistry 287, 28586-28597.

Tanimizu, N., Miyajima, A., 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. Journal of cell science 117, 3165-3174.

Tanimizu, N., Miyajima, A., Mostov, K.E., 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Molecular biology of the cell 18, 1472-1479.

Tanimizu, N., Nakamura, Y., Ichinohe, N., Mizuguchi, T., Hirata, K., Mitaka, T., 2013. Hepatic biliary epithelial cells acquire epithelial integrity but lose plasticity to differentiate into hepatocytes in vitro during development. Journal of cell science 126, 5239-5246.

Tchorz, J.S., Kinter, J., Muller, M., Tornillo, L., Heim, M.H., Bettler, B., 2009. Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. Hepatology 50, 871-879.

Thein, D.C., Thalhammer, J.M., Hartwig, A.C., Crenshaw, E.B., 3rd, Lefebvre, V., Wegner, M., Sock, E., 2010. The closely related transcription factors Sox4 and Sox11 function as survival factors during spinal cord development. Journal of neurochemistry 115, 131-141.

Thomsen, M.K., Francis, J.C., Swain, A., 2008. The role of Sox9 in prostate development. Differentiation; research in biological diversity 76, 728-735.

Tomizawa, M., Garfield, S., Factor, V., Xanthopoulos, K.G., 1998. Hepatocytes deficient in CCAAT/enhancer binding protein alpha (C/EBP alpha) exhibit both hepatocyte and biliary epithelial cell character. Biochemical and biophysical research communications 249, 1-5.

Tremblay, K.D., Zaret, K.S., 2005. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Developmental biology 280, 87-99.

Tsukita, S., Furuse, M., Itoh, M., 2001. Multifunctional strands in tight junctions. Nature reviews. Molecular cell biology 2, 285-293.

Usui, A., Iwagawa, T., Mochizuki, Y., Iida, A., Wegner, M., Murakami, A., Watanabe, S., 2013. Expression of Sox4 and Sox11 is regulated by multiple mechanisms during retinal development. FEBS letters 587, 358-363.

Van Eyken, P., Sciot, R., Callea, F., Van der Steen, K., Moerman, P., Desmet, V.J., 1988. The development of the intrahepatic bile ducts in man: a keratin-immunohistochemical study. Hepatology 8, 1586-1595.

Van, I.S.C., Theard, D., Van Der Wouden, J.M., Visser, W., Wojtal, K.A., Hoekstra, D., 2004. Oncostatin M-stimulated apical plasma membrane biogenesis requires p27(Kip1)-regulated cell cycle dynamics. Molecular biology of the cell 15, 4105-4114.

Vanderpool, C., Sparks, E.E., Huppert, K.A., Gannon, M., Means, A.L., Huppert, S.S., 2012. Genetic interactions between hepatocyte nuclear factor-6 and Notch signaling regulate mouse intrahepatic bile duct development in vivo. Hepatology 55, 233-243.

Vidal, V.P., Chaboissier, M.C., Lutzkendorf, S., Cotsarelis, G., Mill, P., Hui, C.C., Ortonne, N., Ortonne, J.P., Schedl, A., 2005. Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. Current biology : CB 15, 1340-1351.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W., Scherer, G., 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79, 1111-1120.

Wandzioch, E., Zaret, K.S., 2009. Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. Science 324, 1707-1710.

Wang, W., Zhang, J., Zhan, X., Lin, T., Yang, M., Hu, J., Han, B., Hu, S., 2014. SOX4 is associated with poor prognosis in cholangiocarcinoma. Biochemical and biophysical research communications 452, 614-621.

Weinstein, M., Monga, S.P., Liu, Y., Brodie, S.G., Tang, Y., Li, C., Mishra, L., Deng, C.X., 2001. Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. Molecular and cellular biology 21, 5122-5131.

Wilson, M.E., Yang, K.Y., Kalousova, A., Lau, J., Kosaka, Y., Lynn, F.C., Wang, J., Mrejen, C., Episkopou, V., Clevers, H.C., German, M.S., 2005. The HMG box transcription factor Sox4 contributes to the development of the endocrine pancreas. Diabetes 54, 3402-3409.

Wojtal, K.A., Hoekstra, D., van Ijzendoorn, S.C., 2007. Anchoring of protein kinase A-regulatory subunit IIalpha to subapically positioned centrosomes mediates apical bile canalicular lumen development in response to oncostatin M but not cAMP. Molecular biology of the cell 18, 2745-2754.

Wright, E.M., Snopek, B., Koopman, P., 1993. Seven new members of the Sox gene family expressed during mouse development. Nucleic acids research 21, 744.

Yamasaki, H., Sada, A., Iwata, T., Niwa, T., Tomizawa, M., Xanthopoulos, K.G., Koike, T., Shiojiri, N., 2006. Suppression of C/EBPalpha expression in periportal hepatoblasts may stimulate biliary cell differentiation through increased Hnf6 and Hnf1b expression. Development 133, 4233-4243.

Yanai, M., Tatsumi, N., Hasunuma, N., Katsu, K., Endo, F., Yokouchi, Y., 2008. FGF signaling segregates biliary cell-lineage from chick hepatoblasts cooperatively with BMP4 and ECM components in vitro. Developmental dynamics : an official publication of the American Association of Anatomists 237, 1268-1283.

Yimlamai, D., Christodoulou, C., Galli, G.G., Yanger, K., Pepe-Mooney, B., Gurung, B., Shrestha, K., Cahan, P., Stanger, B.Z., Camargo, F.D., 2014. Hippo pathway activity influences liver cell fate. Cell 157, 1324-1338.

Yoder, B.K., Hou, X., Guay-Woodford, L.M., 2002. The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. Journal of the American Society of Nephrology : JASN 13, 2508-2516.

Zaret, K.S., 2008. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nature reviews. Genetics 9, 329-340.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., Jiang, J., 2008. The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Developmental cell 14, 377-387.

Zhang, N., Bai, H., David, K.K., Dong, J., Zheng, Y., Cai, J., Giovannini, M., Liu, P., Anders, R.A., Pan, D., 2010. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Developmental cell 19, 27-38.

Zhang, Y., Guo, X., Xiong, L., Kong, X., Xu, Y., Liu, C., Zou, L., Li, Z., Zhao, J., Lin, N., 2012. MicroRNA-101 suppresses SOX9-dependent tumorigenicity and promotes favorable prognosis of human hepatocellular carcinoma. FEBS letters 586, 4362-4370.

Zhao, B., Li, L., Lei, Q., Guan, K.L., 2010. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes & development 24, 862-874.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., Guan, K.L., 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes & development 21, 2747-2761.

Zhou, R., Bonneaud, N., Yuan, C.X., de Santa Barbara, P., Boizet, B., Schomber, T., Scherer, G., Roeder, R.G., Poulat, F., Berta, P., 2002. SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic acids research 30, 3245-3252.

Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., Stanger, B.Z., 2009. Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727-1739.

2. AIM OF THIS PROJECT

Loss-of-function of SOX9 in liver led to a transient defect in bile duct morphogenesis (Delous et al., 2012; Manfroid et al., 2012). Given the redundancy of SOX factors in cell differentiation, this raised the possibility that other SOX factors are redundant with SOX9 (Kamachi and Kondoh, 2013). SOX4 expression was found to overlap with that of SOX9. Therefore, the aim of this thesis is to characterize the role of SOX4 and to identify the mechanisms of action of SOX4 and SOX9 in bile duct development. This is performed by phenotyping livers from mice with liver-specific inactivation of SOX4 and/or SOX9, and by analyzing cultured hepatoblasts with loss-of-function of SOX4 and/or SOX9.

3. RESULTS

3.1. Transcription factors SOX4 and SOX9 cooperatively control development of bile ducts

This work was initiated by Aline Antoniou. She described the expression of SOX4 in developing liver (Fig. 1 and S1), and made the initial observation that SOX4 and SOX9 were required for prenatal cholangiocyte differentiation (Fig. 2). I characterized the postnatal role of SOX factors (Figs. 3 and 4), as well as the link between SOX factors and polarity (Fig. 5) and signalling pathways (Figs. 6, S2 and S3). Aline Antoniou contributed panels in Figs 3, 5, 6 and S3. I designed Fig. 7 which integrates SOX factors in the biliary gene regulatory network.

Transcription factors SOX4 and SOX9 cooperatively control

development of bile ducts

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Abstract

In developing liver, cholangiocytes derive from the hepatoblasts and organize to form the bile ducts. Earlier work has shown that the SRY-related High Mobility Group box transcription factor 9 (SOX9) is transiently required for bile duct development, raising the question of the potential involvement of other SOX family members in biliary morphogenesis. Here we identify SOX4 as a new regulator of cholangiocyte development. Liver-specific inactivation of SOX4, combined or not with inactivation of SOX9, affects cholangiocyte differentiation, apico-basal polarity and bile duct formation. Both factors cooperate to control the expression of mediators of the Transforming Growth Factor-β, Notch, and Hippo-Yap signaling pathways, which are required for normal development of the bile ducts. In addition, SOX4 and SOX9 control formation of primary cilia, which are known signaling regulators. The two factors also stimulate secretion of laminin α5, an extracellular matrix component promoting bile duct maturation. We conclude that SOX4 is a new regulator of liver development and that it exerts a pleiotropic control on bile duct development in cooperation with SOX9.

Introduction

In developing liver, the hepatocyte and biliary (cholangiocyte) lineages arise from common progenitors called hepatoblasts. The earliest biliary cells start to express the SRY-related High Mobility Group box transcription factor 9 (SOX9) and are detected in mice at embryonic day (E)11.5 in hepatoblasts at the vicinity of the portal vein mesenchyme (Antoniou et al., 2009). When a growing number of hepatoblasts differentiate to cholangiocyte precursors, the latter progressively form the ductal plate, which is constituted of a single layer of SOX9-positive cells surrounding the branches of the portal vein. Primitive ductal structures (PDS) form locally within the ductal plate around E15.5. They are detected as lumina lined on their portal side by SOX9-positive cholangiocyte precursors and on their parenchymal side by SOX9-negative hepatoblasts (Antoniou et al., 2009). During maturation of PDS to bile ducts, all cells lining the ducts acquire their typical cholangiocyte morphology and function. The ducts become surrounded by extracellular matrix (ECM) and mesenchyme, allowing postnatal development of hepatic artery branches (Clotman et al., 2003; Raynaud et al., 2011a). Ductal plate cells that are not involved in bile duct formation transdifferentiate and give rise to periportal hepatocytes, and to cells lining the canals of Hering (Carpentier et al., 2011).

A longstanding effort to characterize the mechanisms of biliary development uncovered essential signaling pathways in cholangiocyte differentiation and bile duct morphogenesis (Lemaigre, 2009; Si-Tayeb et al., 2010). These include the Transforming Growth Factor-β (TGF-β), Notch, and Yap/Hippo pathways (Clotman et al., 2005; Decaens et al., 2008; Geisler et al., 2008; Tan et al., 2008; Tanimizu and Miyajima, 2004; Tchorz et al., 2009; Zhang et al., 2010; Zong et al., 2009). In addition, gene knockout studies identified several transcription factors which are required for normal biliary differentiation and morphogenesis (Lemaigre, 2009).

We previously showed that SOX9 controls the timing of bile duct morphogenesis. Mice with liver-specific inactivation of SOX9 show delayed maturation of PDS into bile ducts (Antoniou et al., 2009), raising the possibility that other member of the SOX factor family can at least partially compensate for the absence of SOX9. Here, we identify SOX4 as a new player in liver development and describe how it regulates bile duct development cooperatively with SOX9.

Results

Biliary-specific expression of SOX4 in developing liver

The mild and transient biliary phenotype resulting from the lack of SOX9 in liver (Antoniou et al., 2009), combined with the known redundancy of SOX factors in cell differentiation (Kamachi and Kondoh, 2013), suggested that other SOX factors control biliary development. RT-qPCR analysis of RNA from total developing liver detected mRNA of several SOX factors (data not shown), and *in situ* hybridization experiments identified SOX4 as the most promising candidate for regulation of biliary development. Indeed, SOX4 was detectable in the ductal plate and developing bile ducts starting at the onset of biliary development (E12.5; Fig. 1A). SOX4 was also expressed in the portal mesenchyme and in rare scattered parenchymal cells; the latter did not belong to the hepatic lineage (see below). Except for the expression in the portal mesenchyme and parenchymal cells, SOX4 expression overlapped with that of SOX9 (Fig. 1B). This expression profile prompted us to investigate the function of SOX4 and to evaluate potential redundancy between SOX4 and SOX9 in biliary development.

For this purpose, we inactivated floxed *Sox4* and/or *Sox9* alleles (Kist et al., 2002; Penzo-Mendez et al., 2007) in developing liver using Cre recombinase driven by albumin and α-fetoprotein gene regulatory regions (*Alfp*-Cre). This approach enables gene inactivation in hepatoblasts prior to the onset of biliary development (Kellendonk et al., 2000). Combined deletion of *Sox4* and *Sox9* was also performed. The efficiency of *Sox4* gene inactivation was controlled by *in situ* hybridization: $Sox4^{loxP/loxP}$;*Alfp*-Cre embryos (Sox4ko) showed inactivation of *Sox4* in the developing cholangiocytes starting at the onset of biliary development (Fig. 1B and S1), while periportal and parenchymal SOX4 expression was not affected. $Sox9^{loxP/loxP}$;*Alfp*-Cre embryos (Sox9ko) showed *Sox9* inactivation in developing ducts (Antoniou et al., 2009). Inactivation of either *Sox* gene did not affect expression of the other (Fig. 1B and Fig. S1).

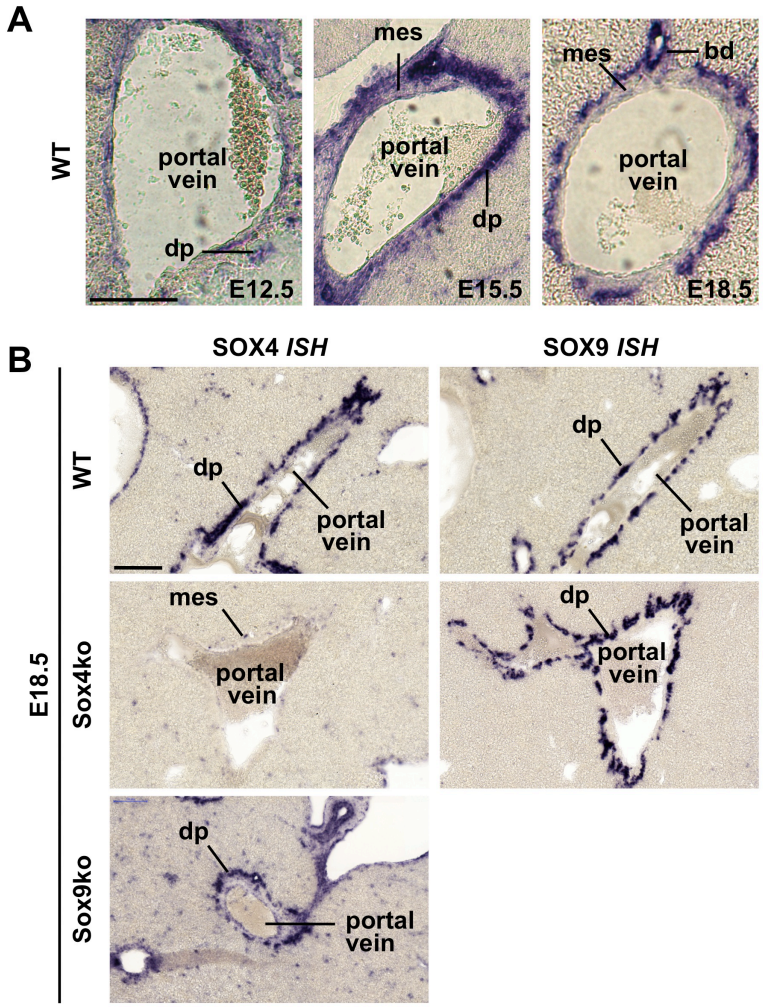

Figure 1. SOX4 and SOX9 expression in the developing liver. (A) *In situ* hybridization in WT livers shows SOX4 mRNA expression in portal mesenchyme and biliary cells starting at E12.5. **(B)** Adjacent tissue sections show overlapping expression of SOX4 and SOX9 in biliary cells. In *Alfp-Cre;Sox4*$^{loxp/loxp}$ (Sox4ko) embryos, SOX4 is deleted in biliary cells but persists in periportal and scattered parenchymal cells; the latter are likely not derived from hepatoblasts. Inactivation of *Sox4* or *Sox9* did not affect expression of the other. mes, mesenchyme; dp, ductal plate; bd, bile duct. Size bar, 100 μm.

SOX4 and SOX9 control cholangiocyte differentiation

PDS are detected in wild-type embryos starting at E15.5, and consist of cells expressing the hepatoblast marker Hepatocyte Nuclear Factor 4 (HNF4) on the parenchymal side and HNF1β predominantly on the portal side. The absence of SOX4 or SOX9 induced only limited differentiation defects at that stage, namely persistent expression of HNF4 in some cells lining the portal side of PDS and lower expression of HNF6 in differentiating cholangiocytes (Fig. 2A). In contrast, the combined inactivation of both factors (*Sox4*$^{loxP/loxP}$;*Sox9*$^{loxP/loxP}$;*Alfp*-Cre (Sox4/Sox9ko)) strongly perturbed biliary differentiation: biliary markers (HNF6, HNF1β) were barely detectable and expression of the hepatoblast marker HNF4 persisted in cells lining small-sized lumina (Fig. 2A). These data were in line with our observations on hepatoblasts purified from E12.5 livers. Indeed, when these hepatoblasts are grown in conditions promoting biliary gene expression, biliary genes are upregulated and hepatocyte markers are decreased after 72 h of culture. In contrast, hepatoblasts purified from Sox4/Sox9ko livers showed lower biliary gene induction and lower repression of hepatocyte markers (Fig. S2).

Later, at the end of gestation (E18.5), wild-type PDS had matured to generate bile ducts symmetrically lined by differentiated cholangiocytes (Fig. 2B). In contrast, Sox4ko, Sox9ko and Sox4/Sox9ko cholangiocyte differentiation had not markedly progressed as compared to E15.5. This was illustrated by the persisting expression of HNF4 on the parenchymal side of the bile ducts in Sox4ko and Sox9ko, and in all cells lining the lumen in Sox4/Sox9ko. Also, HNF1β expression was low in all livers deficient in SOX4 and/or SOX9, as compared to wild-type (Fig. 2B).

After birth at P6, cholangiocyte differentiation had returned to normal in Sox4ko and Sox9ko livers, as shown by the expected downregulation of HNF4 and increased expression of HNF6 in cholangiocytes (Fig. 3). Therefore, the absence of SOX4 alone led to delayed cholangiocyte differentiation, as found earlier in the absence of SOX9 (Antoniou et al., 2009). In contrast, Sox4/Sox9ko mice showed persistent and pronounced biliary differentiation defects. In the absence of the two SOX factors, high levels of the hepatoblast/hepatocyte marker HNF4 were detected in cells lining lumina and HNF6 expression was low. In addition, these cells displayed characteristics of periportal hepatocytes as indicated by detection of carbamoyl phosphate synthase I (CPS1), an enzyme normally expressed in zone 1 and 2 hepatocytes (Christoffels et al., 1999). Glutamine synthase, an enzyme of perivenous hepatocytes was not expressed in the cells lining lumina (Fig. 3).

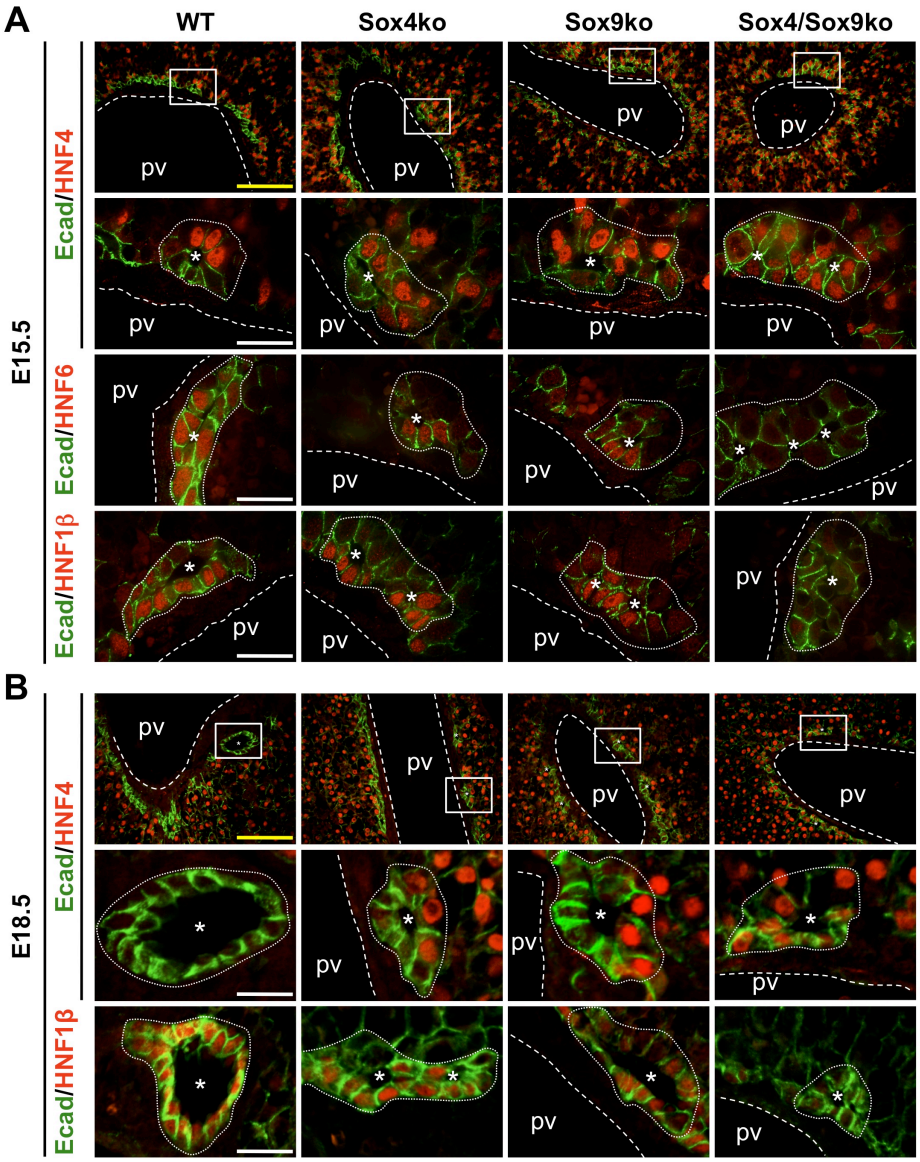

Figure 2. **Cholangiocyte differentiation is perturbed in the absence of SOX4 and SOX9. (A)** At E15.5, immunostaining shows residual expression of HNF4 in the portal side of Sox4ko and Sox9ko PDS as well as reduced expression of HNF6. In Sox4/Sox9ko, HNF6 and HNF1β expression was not detected while HNF4 persisted in all PDS cells. **(B)** At E18.5, asymmetrical bile ducts with persistent expression of HNF4 and low expression of HNF1β are detected in Sox4ko and Sox9ko; this phenotype is worsened in Sox4/Sox9ko. Ecad; E-cadherin; pv, portal vein; *, lumen of developing bile ducts. White size bar, 25 μm; yellow size bar, 100 μm.

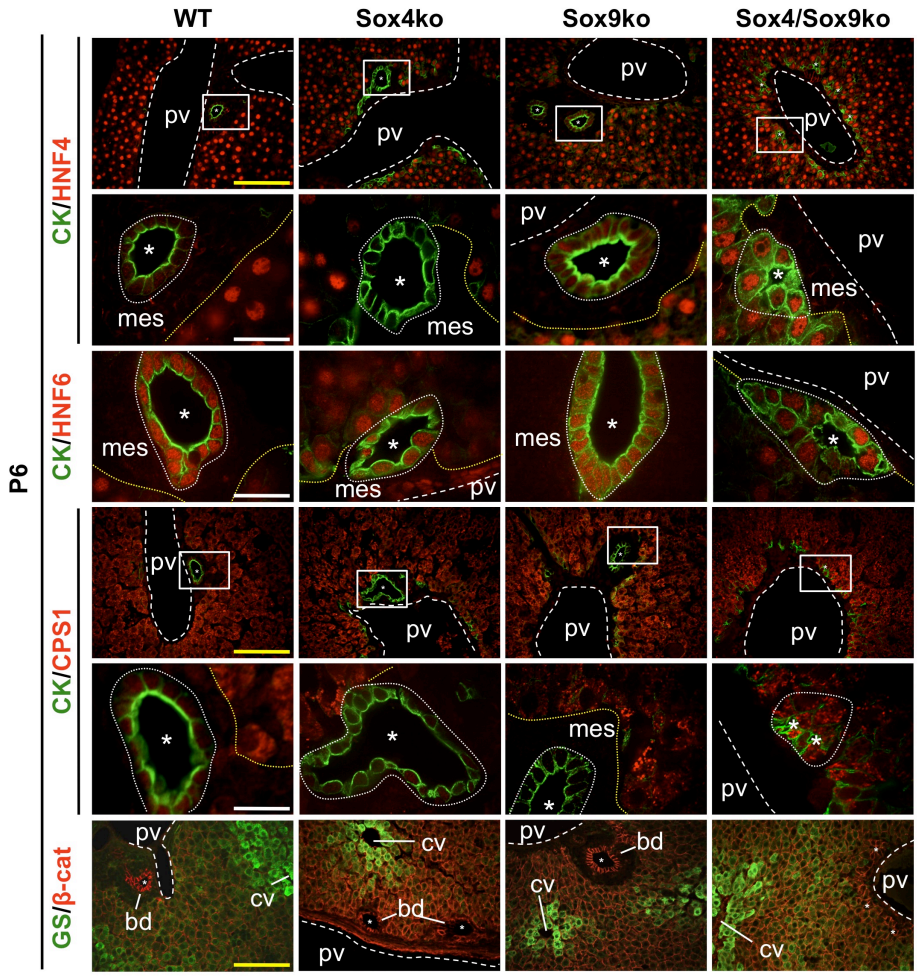

Figure 3. The lack of SOX4 or SOX9 induces transient defects in bile duct development, while combined absence of both factors is associated with persisting anomalies. Immunostaining at P6 shows normal expression of the differentiation markers HNF4 and HNF6 in Sox4ko and Sox9ko livers. Biliary cells in Sox4/Sox9ko liver display hepatocyte characteristics, as shown by expression of HNF4 and carbamoyl phosphate synthase I (CPSI), and low levels of HNF6. Expression of glycogen synthase (GS) was normal and strictly pericentral. β-cat, β-catenin; CK, pan-Cytokeratin; pv, portal vein; mes, mesenchyme; bd, bile duct; cv, central vein; *, lumen of bile ducts. White size bar, 25 μm; yellow size bar, 100 μm.

In 6-week-old adults, the Sox4/Sox9ko mice were cholestatic, as shown by the high levels of serum bilirubin (Fig. 4A). This was associated with liver fibrosis and ductular reactions characterized by luminal structures lined by cells expressing high levels of cytokeratin (pan-cytokeratin immunostaining), and variable levels of HNF4 (Fig. 4B). Retrograde injection of ink in the biliary tree demonstrated that the bile ducts had not developed properly: hilar ducts appeared dilated and truncated and diffusion of ink into small-sized channels indicated that the lumina of ductular reactions were connected to the hilar ducts.

We concluded that SOX4 and SOX9 individually control the timing of cholangiocyte differentiation and that they are cooperatively required for cholangiocyte differentiation.

SOX factors control polarity of cholangiocytes

Polarization and differentiation are coordinated in epithelia (Kesavan et al., 2009) and are essential for cholangiocyte function. Therefore, we investigated the acquisition of polarity in biliary cells in SOX-mutant biliary cells.

In developing liver, the apical markers Osteopontin (OPN) and Mucin-1 (Muc) became detectable at the apical pole of wild-type PDS cells starting at E15.5. In SOX4 or SOX9-deficient embryos, OPN was initially cytoplasmic or undetectable (E15.5) but became detectable at the apical pole at the end of gestation (E18.5); Mucin-1 was apical, as expected, but low at E18.5. In contrast, the lack of both SOX factors was associated with absence of apical polarization during gestation (Fig. 5).

In the postnatal period (P6), polarization of SOX9-deficient livers progressively normalized (Fig. 5), confirming our earlier observations (Antoniou et al., 2009). In contrast, in the absence of SOX4, polarity remained perturbed postnatally (Fig. 5): at the apical pole, OPN was mislocalized and Ezrin and Mucin-1 were expressed at low levels; at the basal pole, laminin expression was fragmented. In addition, SOX4-deficient biliary cells were no longer cuboidal but instead displayed an irregular cobblestone-like shape. Importantly, the combined absence of SOX4 and SOX9 was associated with major polarity defects: apical markers (OPN, Mucin-1, Ezrin) and basal markers (pan-laminin) were not detected and cell morphology was irregular; pan-cytokeratin staining further indicated that the cytoskeleton was disrupted (Fig. 5).

Therefore, Sox4 is essential to establish apico-basal polarity and cell morphology, whereas Sox9 is only transiently required for polarization; both factors cooperate to set up apico-basal polarity.

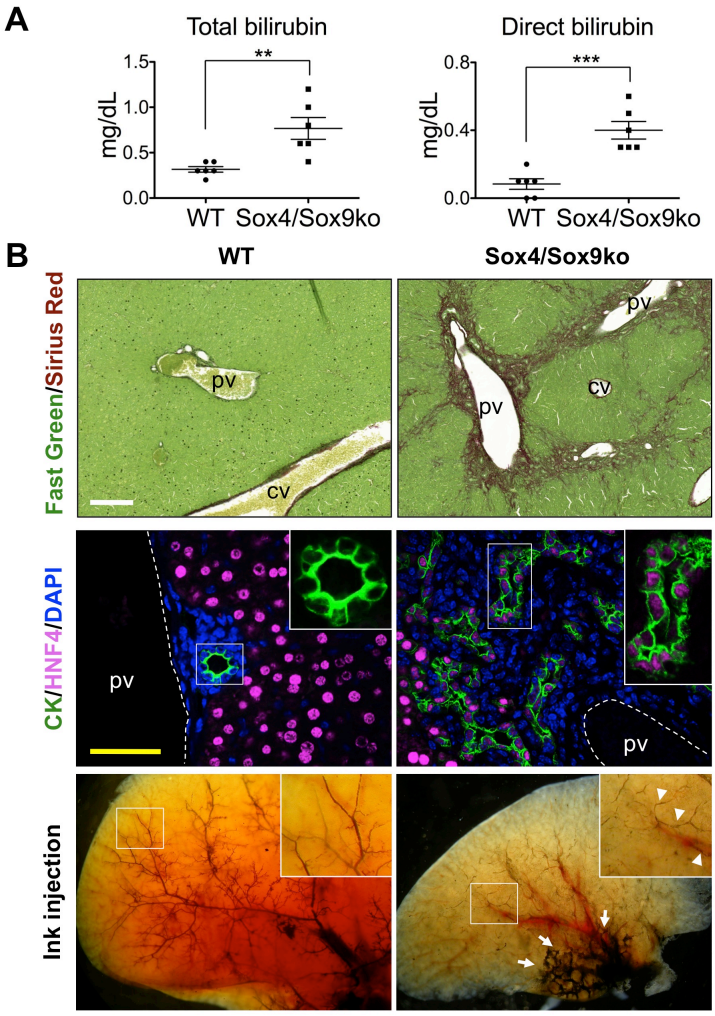

Figure 4. Six-week-old mice with liver-specific inactivation of SOX4 and SOX9 are cholestatic. (A) Serum levels of bilirubin are increased in Sox4/Sox9ko mice (data are means ± SD; n= 6; **p < 0.01, ***p < 0.001, non-paired t-test). **(B)** Septal fibrosis and ductular reactions develop as a result from cholestasis caused by abnormal development of bile ducts: retrograde injection of ink reveals truncated and dilated hilar ducts (arrows), and ductular reactions connected with the hilar ducts (arrowheads in inset). pv, portal vein; cv, central vein. White size bar, 200 μm; yellow size bar, 50 μm.

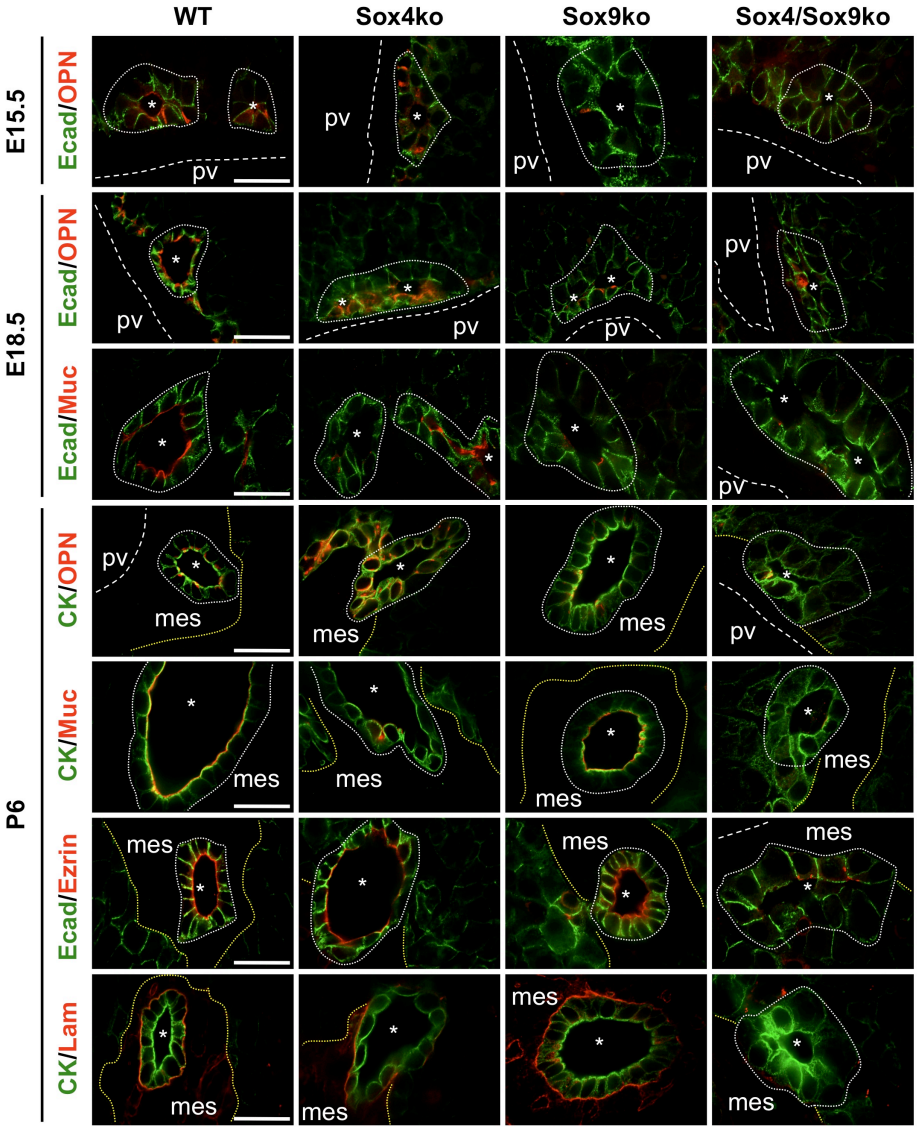

Figure 5. SOX factors control polarity of cholangiocytes. Immunostaining shows downregulation of apical polarity markers Osteopontin (OPN) and Mucin1 (Muc) in Sox4ko and Sox9ko at E15.5 and E18.5. Polarity marker expression normalizes after birth (P6) in Sox9ko, but not in Sox4ko ducts which display mislocalization of OPN, downregulation of Mucin1 and Ezrin at the apical pole, and fragmented laminin (Lam) staining at the basal pole. In Sox4/Sox9ko, all polarity markers were absent or barely detected at all stages tested. Cell morphology is perturbed as shown by Cytokeratin (CK) staining in Sox4ko and Sox4/Sox9ko. Ecad, E-cadherin; pv, portal vein; mes, mesenchyme; *, lumen of bile ducts. Size bar, 25 μm.

SOX4 and SOX9 control signaling pathways involved in biliary development

Cholangiocyte differentiation and bile duct morphogenesis are induced by a combination of intercellular signaling pathways, the best characterized being TGF-β, Notch, and Hippo pathways. SOX4 and SOX9 are known regulators of these pathways in cancer and development (Bhattaram et al., 2010; Delous et al., 2012; Kuwahara et al., 2012; Manfroid et al., 2012; Moreno, 2010; Scharer et al., 2009; Song et al., 2013; Vervoort et al., 2013), raising the possibility that SOX factors control biliary development by modulating the response to intercellular signaling. To address this hypothesis, we investigated the expression of signaling markers in SOX factor-deficient livers.

Transforming growth factor β receptor II (TβRII) mediates the TGF-β response in developing hepatoblasts (Clotman et al., 2005; Takayama et al., 2014). When the hepatoblasts are stimulated by TGF-β, the cells differentiate to the cholangiocyte lineage in which TβRII then becomes repressed (Antoniou et al., 2009). Therefore, in wild-type developing ducts, repression of TβRII is a mark of previously active TGF-β signaling: at E15.5 TβRII was repressed on the portal side of PDS, but still detectable on their parenchymal side; at E18.5, when ducts have matured, TβRII was no longer detected in cholangiocytes (Fig. 6). In the absence of SOX4 and/or SOX9, TβRII was not repressed on the portal side of PDS at E15.5. At E18.5, TβRII is normally repressed in Sox9ko cholangiocytes while it was still detected in some duct cells in the absence of SOX4 and in double knockouts (Fig. 6). This was in line with our observations on cultured Sox4/Sox9ko hepatoblasts: when grown in culture as above, expression of TβRII and of PAI-1, a TGF-β signaling target, increases in the absence of SOX4 and SOX9 (Fig. S2). These data indicate that SOX4 and SOX9 control TGF-β signaling early in biliary development.

Notch signaling is critical in biliary development and Hes1 induction is a marker of active Notch signaling. The absence of SOX4 and/or SOX9 did not affect Hes1 expression at E15.5 (Fig. S3A). However, at E18.5 Hes1 was detected at lower levels in most SOX-mutant cells as compared to wild-type cells (Fig. 6). Abnormal Hes1 expression persisted postnatally in the combined absence of SOX4 and SOX9 (Fig. S3A). Therefore, SOX4 and SOX9 control Notch signaling in developing biliary cells, but this effect was detectable at a later stage than that on TGF-β signaling.

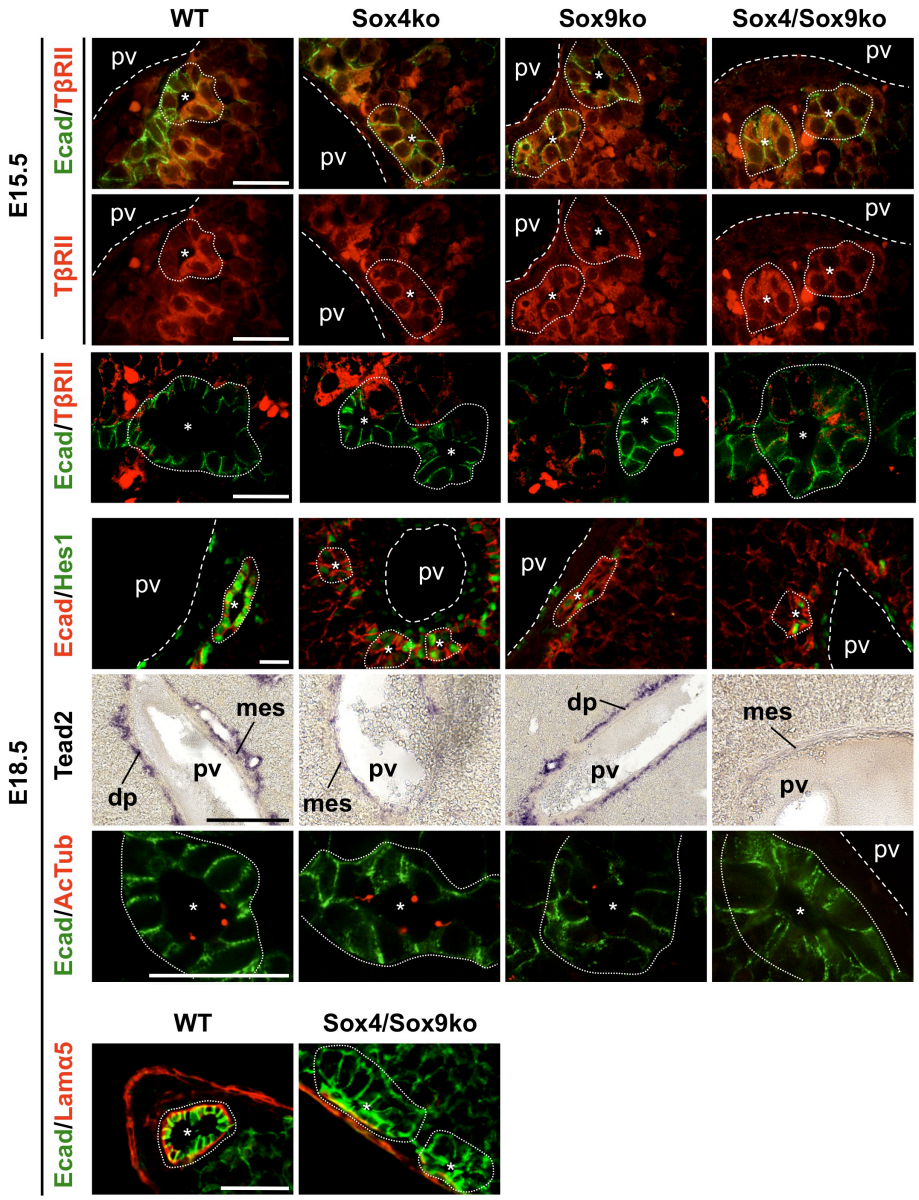

Figure 6. SOX factors control signaling pathways involved in biliary development. At E15.5, TβRII is repressed on the portal side of PDS in wild-type livers but persists in all PDS cells of knockout mice. At E18.5, TβRII is downregulated in wild-type ducts but still detectable in a subset of cells lining Sox4ko and Sox4/Sox9ko ducts. In the absence of SOX4 and/or SOX9, Hes1 is low or absent from cholangiocytes. Tead2 is expressed in the ductal plate and portal mesenchyme (*in situ* hybridization) and biliary-specific expression depends on SOX4. Cilia (acetylated tubulin; AcTub) are absent in Sox4/Sox9ko ducts.

Laminin α5 (Lamα5) staining surrounds bile ducts of wild-type mice while its expression is restricted along the portal side of bile ducts of Sox4/Sox9ko. Ecad, E-cadherin; pv, portal vein; dp, ductal plate; mes, mesenchyme; *, lumen of bile ducts. White size bar, 25 μm; black size bar, 200 μm.

The Hippo-Yap pathway is required for biliary development, and Tead2, the main co-activator of Yap, is a direct target of SOX4 in neural and mesenchymal progenitors (Bhattaram et al., 2010; Zhang et al., 2010). We found that Yap is predominantly expressed in biliary cells. Yap expression did not differ signficantly in wild-type and Sox mutant mice (Fig. S3B). However, Tead2, which is detected in biliary cells, was absent from SOX4-deficient cholangiocytes, indicating that Tead2 is regulated by SOX4 in biliary cells (Fig. 6). This was also seen in cultured hepatoblasts deficient in SOX4 and SOX9 (Fig. S2). In contrast, SOX9 did not seem to control Tead2 (Fig. 6).

The apical pole of cholangiocytes has a primary cilium, and ciliary defects are associated with perturbed biliary development (Raynaud et al., 2011b). In addition, cilia integrate several signaling pathways required for biliary development such as Notch, TGF-β and Hippo pathways (Clement et al., 2013; Habbig et al., 2011; Leitch et al., 2014b). Cilia were detected by staining for acetylated tubulin in livers deficient in SOX4 or SOX9 at prenatal and postnatal stages (Fig. 6 and Fig.S3A). In contrast, the combined absence of SOX4 and SOX9 prevented prenatal development of cilia, and led to a reduced number of cilia postnatally (Fig. 6 and Fig. S3A), suggesting that aberrant cilium development further contributes to abnormal bile duct development.

The ECM surrounding developing bile ducts contains laminin α1 and α5. While laminin α1, produced by adjacent fibroblasts, is essential to establish apicobasal polarity in cholangiocytes, laminin α5 is secreted by biliary cells and is required for maturation of the ducts (Tanimizu et al., 2012). The combined absence SOX4 and SOX9 did not perturb expression of laminin α1, but led to the lack of laminin α5 on the parenchymal side of bile ducts (Fig. 6). The lack of laminin α5 in the absence of SOX factors is further expected to induce deficient bile duct development.

We conclude that SOX4 and SOX9 cooperatively control bile duct development and that their expression is required for normal expression of mediators of TGF-β, Notch, and Hippo signaling. In addition, cilia formation at the apical pole and secretion of laminin α5 at the basal pole, two processes involved in normal duct morphogenesis, are also dependent on SOX factors.

Discussion

In previous work, we have shown that SOX9 is one of the earliest and most specific biliary markers. The analysis of SOX9-deficient livers revealed that this factor controls the timing of bile duct development (Antoniou et al., 2009). Here, we found that SOX4 is also expressed in developing cholangiocytes; its inactivation delays biliary differentiation and is associated with deficient apico-basal polarity and perturbed cell morphology. In addition, the two factors cooperate in bile duct development, since their combined absence inhibits differentiation, polarization and bile duct morphogenesis. Analysis of signaling effectors and mediators in SOX4- and SOX9-deficient livers suggest that the two factors modulate the function of several pathways required for bile duct development.

SOX4 and SOX9 belong to distinct groups of the SOX family, called SoxC and SoxE, respectively (Lefebvre et al., 2007). The SoxC group consists of SOX4, SOX11 and SOX12. *In situ* hybridization of SOX11 did not reveal expression in biliary cells, and SOX12 mRNA levels in total liver were one order of magnitude lower than those of SOX4 (data not shown). The SoxE group consists of SOX8, SOX9 and SOX10. mRNAs of SOX8 and SOX10 were only detected at marginal levels in developing liver by qRT-PCR, and were not considered for further investigation. Therefore, SOX4 and SOX9 stood out in developing livers as candidate regulators of biliary development.

SOX factors co-regulating a developmental process often belong to the same group. There are exceptions such as the SoxD proteins SOX5/SOX6 and the SoxE protein SOX9, known as the "Sox trio", which cooperate to control chondrogenesis (Kamachi and Kondoh, 2013; Lefebvre et al., 2007). To our knowledge, biliary development is the first biological process coregulated by a "Sox duo" composed of SOX4 and SOX9.

Combined absence of SOX4 and SOX9 affected several signaling pathways known to control biliary development. Therefore, it is unlikely that the knockout phenotype depends on a small set of genes directly regulated by the two factors. Rather, our data suggest that SOX4 and SOX9 exert a combination of pleiotropic and indirect effects on bile duct development. When considering the timing of the Sox4/Sox9 ko phenotype, abnormal TGF-β signaling appears as an initiating event. At E15.5, expression of TβRII persists in all Sox4/Sox9ko cells lining lumina and the cells display increased TGF-β signaling as evidenced by increased PAI-1 expression. Interestingly, HNF6 is a known repressor of TβRII expression (Clotman et al., 2005; Plumb-Rudewiez et al., 2004) and increased expression of TβRII in

Sox4/Sox9ko cells *in vivo* and in *vitro* correlates with decreased levels of HNF6. These data suggest that regulation of HNF6 by SOX4 and SOX9 is a key process in biliary differentiation.

Reduced expression of Hes1 in developing duct cells unveils perturbed Notch signaling. Abnormal Hes1 expression in the absence of SOX4 and SOX9 is detected at E18.5, *i.e.* at a later stage than abnormal TβRII expression. This raises the possibility that perturbed Notch signaling is a consequence of abnormal initiation of biliary development. Still, Notch activation induces Hes1 and SOX9, and promotes biliary morphogenesis (Kodama et al., 2004; Zong et al., 2009). Moreover, in developing pancreas, SOX9 regulates Hes1, raising the possibility that SOX9 modulates Hes1 activity in bile ducts (Seymour et al., 2007; Shih et al., 2012). Similarly, SOX4 activates Notch signaling in prostate cancer (Moreno, 2010) suggesting that this factor may exert similar functions in cholangiocytes. Therefore, direct effects of SOX4 and SOX9 cannot be excluded in biliary development. Along the same lines, primary cilia are absent at the apical pole of cholangiocytes in liver with combined inactivation of SOX4 and SOX9. Since primary cilia can modulate Notch and TGF-β signaling (Clement et al., 2013; Leitch et al., 2014b), we suggest that altered ciliary function may secondarily contribute to deficient bile duct development via perturbed Notch and TGF-β signaling.

The YAP co-activator Tead2 is controlled by SOX4. Importantly, downregulation of Tead2 is neither associated with changes in the nuclear localization of YAP, nor with downregulation of YAP target genes such as CTGF (data not shown). This raises the possibility that other Tead proteins compensate for the reduced levels of Tead2, or that the SOX4-Tead2 cascade does not modulate bile duct development.

Finally, perturbed laminin deposition may contribute to the biliary phenotype of Sox4/Sox9ko mice. Indeed, SOX9 is required for activation of ECM genes and for laminin deposition (Hanley et al., 2008; Lincoln et al., 2007; Rockich et al., 2013), and laminins regulate the development of bile ducts (Tanimizu et al., 2012). Laminin expression was delayed in the absence of SOX9, permanently fragmented in the absence of SOX4 and missing in the absence of both factors (Fig. 5). Thus, the level of laminin deficiency correlates well with the severity of the biliary phenotype in Sox9ko, Sox4ko and Sox4/Sox9ko mice. In addition, laminins regulate apico-basal polarity (Tanimizu et al., 2007), suggesting that abnormal polarity in SOX-deficient livers results from the reduced levels of laminin α5 at the basal pole. Abnormal polarity may in turn affect partitioning of cytoskeletal proteins

such as cytokeratins whose intracellular distribution is strongly affected in the absence of both SOX4 and SOX9.

In conclusion, we found that SOX factors cooperate to regulate bile duct development and propose that they are integrated in the biliary gene network and exert pleiotropic effects on polarity, differentiation and morphogenesis. These effects are initiated by regulation of TGF-β signaling by the SOX factors. Our work is relevant to improve and validate the *in vitro* production of pluripotent stem cells into cholangiocytes and for potential cell therapy of biliary disease.

Materials and methods

Animals

Sox9$^{loxP/loxP}$, *Sox4*$^{loxP/loxP}$ and albumin/α-fetoprotein-Cre (*Alfp*-Cre) mice have been described (Kellendonk et al., 2000; Kist et al., 2002; Penzo-Mendez et al., 2007) and were kindly provided by G. Scherer, V. Lefebvre and F. Tronche, respectively. Experimental protocols were approved by the Animal Welfare Committee of the Université catholique de Louvain.

Immunodetection and histochemistry

Embryos were formalin-fixed, paraffin-embedded and sectioned at 9 µm. Sections were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval, sections were microwave-heated for 10 min in 10 mM sodium citrate (pH 6.0). Sections were permeabilized for 10 min with 0.3% Triton X-100 in phosphate-buffered saline (PBS), blocked for 45 min with 3% milk/10% bovine serum albumin (BSA)/0.3% Triton X-100 in PBS, and incubated overnight at 4°C with primary antibodies (listed in supplementary Table 1) diluted in 3% milk/10% BSA/0.3% Triton X-100 in PBS. Secondary antibodies (listed in supplementary Table 2) were diluted 1:1000 in 10% BSA/0.3% Triton X-100 in PBS and incubated on tissue sections for 1 hour at 37°C.

Immunodetections of Hairy and enhancer of split-1 (Hes1) and Acetylated-Tubulin were carried out with Tyramide Signal Amplification kit (#T-20935, Molecular probes, Life technologies). The protocol was adapted for detection of laminin α5 staining: embryos were gelatin-embedded after formalin-fixation, and the antigen retrieval step was omitted.

Immunohistochemical detection was performed with 3,3'-Diaminobenzidine chromogen (DAB, #K3468, Dako) followed by haematoxylin counterstaining.

For collagen deposit staining, tissue sections were incubated for 5 h at room temperature in picric acid solution (#92540, Sigma-Aldrich) complemented with Fast Green dye (1g/L) (#F7258, Sigma-Aldrich) and with Direct Red 80 dye (1g/L) (#365548, Sigma-Aldrich).

Whole slides were scanned using the MIRAX scan system (Zeiss). Immunofluorescently-labeled sections were analysed using a Zeiss Cell Observer Spining Disk confocal microscope. Since bile duct development progresses from

the hilum to the periphery of the liver lobes, all analyzed sections were made near the hilum.

In situ hybridization

Embryos were fixed overnight at 4°C in ethanol 60%, formaldehyde 30% and acetic acid 10%, paraffin-embedded and sectioned (9 µm). Tissue sections were deparaffinized in xylene and hydrated in graded alcohols. Tissue sections were hybridized overnight at 65°C with digoxygenin-labeled antisense RNA probes for Sox4, Sox9 (kindly provided by M. Sander) and Tead2 (Jacquemin et al., 1996) diluted in hybridization solution (formamide 50%, dextran sulfate 50% solution 20X (#S4030, Millipore), tRNA (1mg/mL) (#10109517001, Roche), Denhardt's solution 50X (#750018, Invitrogen, Life technologies)). Tissue sections were blocked for 1 h at room temperature in Tris buffered saline 1% Tween 20 (TBST) complemented with blocking reagent 1.5% (#11096176001, Roche), and incubated overnight at 4°C with an alkaline phosphatase-coupled antidioxygenin antibody diluted 1:1500 (#11093274910, Roche), and then processed for alkaline phosphatase activity with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (#11681451001, Roche).Whole slides were scanned using the MIRAX scan system (Zeiss).

Cell culture

Sox4$^{loxP/loxP}$; *Sox9*$^{loxP/loxP}$ females were mated with *Sox4*$^{loxP/loxP}$; *Sox9*$^{loxP/loxP}$; *Alfp*-Cre males. E12.5 embryos were dissected and genotyped. Livers were collected and cell-dissociated in RPMI (#31870-025, Gibco, Life technologies) containing collagenase IV (1mg/mL) (#43E14253, Worthington), dispase (1mg/mL) (#17105-041, Gibco, Life technologies) and DNase I (0.1mg/mL) (#11284932001, Roche). Cells were resuspended in 40 µl of EDTA 2 mM, 0.5% BSA in PBS (MACS buffer) and incubated with 2 µl goat biotinylated anti-Dlk1 antibody (# BAF1144, R&D systems) for 10 min at 4°C to isolate Dlk1-positive hepatoblasts. After washing with PBS, cells were resuspended in 80 µl of MACS buffer, and 20 µl of anti-biotin microbeads (#120-000-900, Miltenyi) were added followed by 15 min incubation at 4°C. After washing with MACS buffer, Dlk1-positive hepatoblasts were purified using a magnetic column (#130-042-201, Miltenyi), plated in 24-well plates coated with collagen (#08-115, Millipore) (50.000 cells/well) and cultured for 72 h in RPMI supplemented with 10% fetal bovine serum (#F6178, Sigma-Aldrich), EGF (50 ng/mL) (#AF-100-15, Peprotech), ITS 100X (#41400-045, Gibco, Life technologies) and IGF-II (30 ng/mL) (#100-12, Peprotech). Total RNA was extracted and gene expression was quantified by RT-qPCR with primers listed in Supplementary Table 3.

Biliary tree casting

Six- to eight-week-old mice were euthanized. Ink (Drawing ink A, Pelikan, Germany) was injected in canulated common bile duct (Walter et al., 2012). The entire liver was removed, formalin-fixed and clarified in a 1:2 solution of benzyl alcohol and benzyl benzoate.

Serum analysis

Blood was collected from 6- to 8-week-old mice and serum was analyzed using reagents for total and direct bilirubin (#442745, #439715, synchron LX (clinical system, Beckman Coulter).

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Supplementary Table 1: List of primary antibodies used for immunostaining experiments

Primary antibody	Species	Dilution	Source	Catalog number
HNF4	goat	1/50	Santa Cruz	sc-6556
E-cadherin	mouse	1/200	BD Biosciences	610182
CK, pan-cytokeratin	mouse	1/1000	Sigma	c5992
Lam, pan-Laminin	rabbit	1/50	Sigma	L9393
Osteopontin	goat	1/100	R&D Systems	AF808
Sox9	rabbit	1/500	Chemicon	AB5535
Mucin 1	hamster	1/200	Neomarkers	HM-1630-P
Ac-tubulin	mouse	1/5000	sigma	T6793
TβRII	rabbit	1/50	Abcam	ab28382
HNF6	rabbit	1/50	Santa Cruz	sc-13050
HNF1β	rabbit	1/25	Santa Cruz	sc-22840
Hes1	rabbit	1/1500	B. Stanger lab	-
Ezrin	mouse	1/200	Neomarkers	MS661P1
β-catenin	mouse	1/1000	BD Biosciences	610154
GS	mouse	1/2500	BD Biosciences	610517
CPS1	rabbit	1/100	Abcam	ab3682
Cytokeratin 19	rat	1/50	DSHB	TROMA-III
Yap	rabbit	1/100	Cell signaling	4912
Laminin α5	rabbit	1/2500	Takako Sasaki	1121+

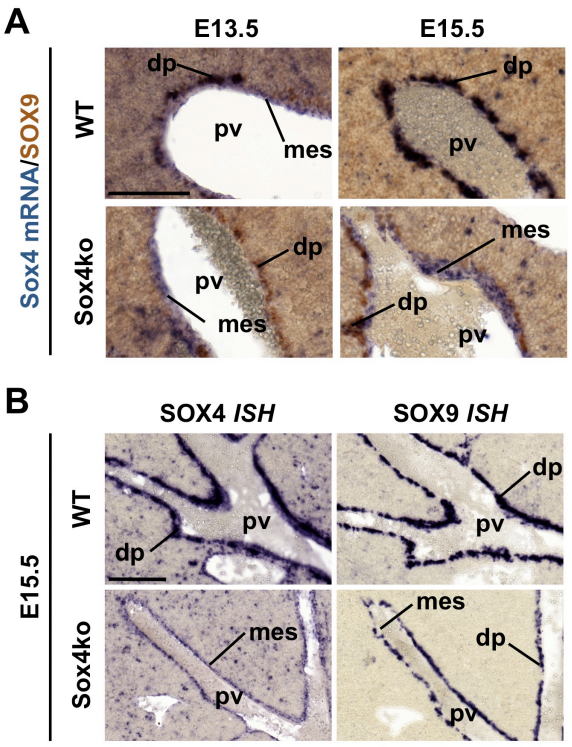
Supplementary Table 2: List of secondary antibodies used for immunostaining experiments

Secondary antibody	Species	Source	Catalog number
Alexa Fluor 488 anti-mouse IgG1	goat	Invitrogen	A-21121
Alexa Fluor 594 anti-mouse IgG2a	goat	Invitrogen	A-21135
Alexa Fluor 488 anti-mouse (H+L)	donkey	Invitrogen	A-21202
Alexa Fluor 594 anti-mouse (H+L)	donkey	Invitrogen	A-21203
Alexa Fluor 594 anti-goat	donkey	Invitrogen	A-11058
Alexa Fluor 647 anti-goat	donkey	Invitrogen	A-21447
Alexa Fluor 594 anti-rabbit	donkey	Invitrogen	A-21207
Biotinylated anti-hamster	goat	JacksonImmuno	127-065-160
HRP anti-rat	chicken	Santa Cruz	SC2956

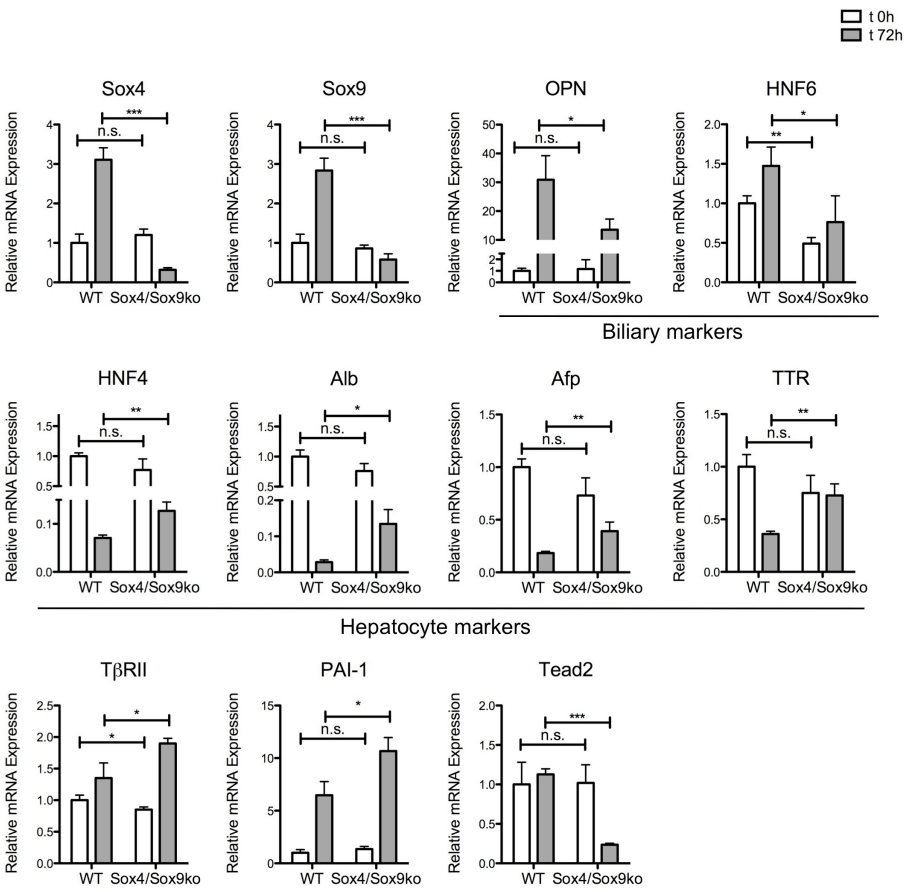
Supplementary Table 3: List of primer sequences used for RT-qPCR

Gene	Pimer sequences (5'-3')
Actin	Fw - TCCTGAGCGCAAGTACTCTGT Rv - CTGATCCACATCTGCTGGAAG
Sox4	Fw - GAACGCCTTTATGGTGTGGT Rv - GAACGGAATCTTGTCGCTGT
Sox9	Fw - CAAGACTCTGGGCAAGCTCTG Rv - TCCGCTTGTCCGTTCTTCAC

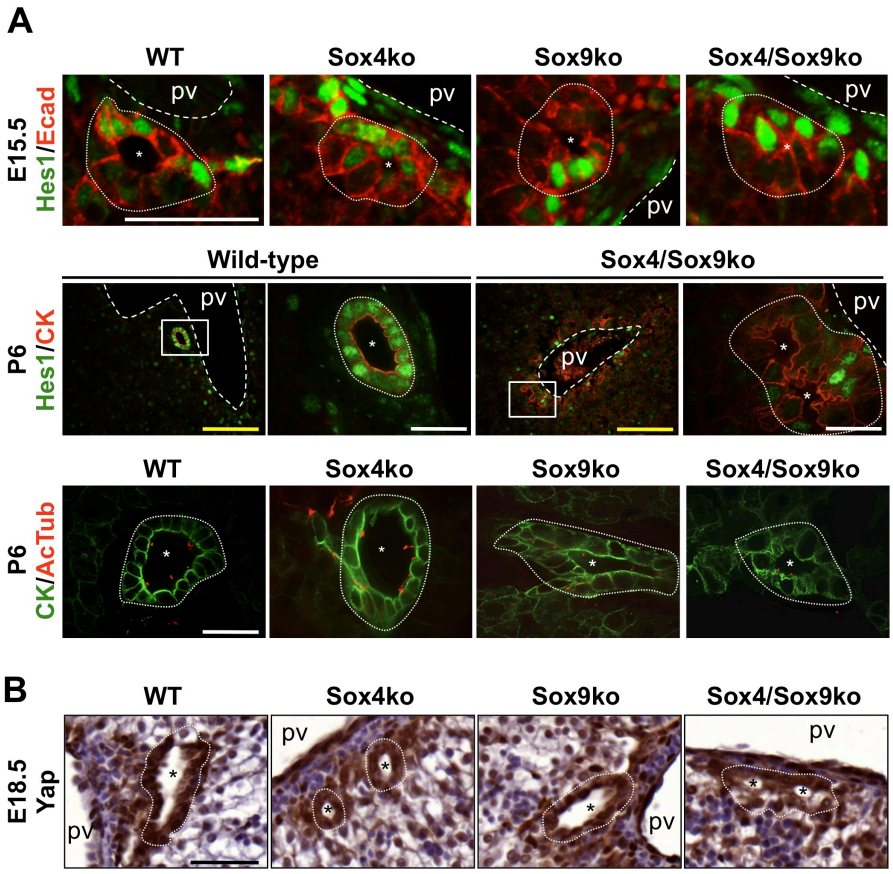
Osteopontin (OPN)	Fw - TGAGATTGGCAGTGATTTGC Rv - CTGCTTCTGAGATGGGTCAG
HNF6	Fw - TTCCAGCGCATGTCGGCGCTC Rv - GGTACTAGTCCGTGGTTCTTC
HNF4	Fw - AGCTCGAGGCTCCGTAGTGTTT Rv - GAAAATGTGCAGGTGTTGACCA
Albumin (Alb)	Fw - GCTGAGACCTTCACCTTCCA Rv - TCTTCAGTTGCTCCGCTGTA
α-fetoprotein (Afp)	Fw - GGAGGCTATGCATCACCAGT Rv - CATGGTCTGTAGGGCTTTGC
Transthyretin (TTR)	Fw - GGGCTCACCACAGATGAGAA Rv - CAGAGTCGTTGGCTGTGAAA
TβRII	Fw - CGGAAATTCCCAGCTTCTGG Rv - TTTGGTAGTGTTCAGCGAGC
PAI-1	Fw - TGGCTCAGAGCAACAAGTTC RV - GCAGTTCCACGACGTCATACTCG
Tead2	Fw - AGCTGAGTGAGCTGGGTTTGAGTT Rv - AGCAGGAAGGTTACAAGGCAGTCA



Supplementary Figure 1. SOX4 is absent in biliary cells of Sox4ko livers starting at the onset of biliary development. (A) Immunolabeling of SOX9 (brown) and *in situ* hybridization (*ISH*) of SOX4 (purple). Inactivation of SOX4 does not affect SOX9 expression. (B) *In situ* hybridization of SOX4 and SOX9. Alfp-Cre-mediated inactivation of *Sox4*$^{flox/flox}$ alleles suppresses *Sox4* expression in biliary cells and not in portal mesenchymal cells. mes, mesenchyme; dp, ductal plate; pv, portal vein. Size bar, 100 μm.



Supplementary Figure 2. SOX factors induce expression of biliary markers and repress expression of hepatocyte markers. After 72 h of culture, hepatoblasts purified from WT embryos show increased biliary gene expression and decreased hepatocyte gene expression. Expression of signal transducers is modulated by SOX factors. Mean ± SD. n=3. *p< 0.05; **p< 0.01; ***p< 0.001 ; non-paired t-test; n.s, non-significant. OPN, osteopontin; Alb, Albumin; Afp, α-fetoprotein; TTR, Transthyretin.



Supplementary Figure 3. SOX factors control signaling mediators involved in biliary development. (A) In the absence of SOX factors, Hes1 expression in developing cholangiocytes is normal at E15.5, but is reduced at P6. The number of cilia (acetylated tubulin, AcTub) is reduced in Sox4/Sox9ko cholangiocytes at P6. (B) The expression and nuclear location of Yap in cholangiocytes is not affected by the absence SOX4 and/or SOX9. CK, pan-cytokeratin; pv, portal vein; *, lumen of bile ducts. White and black size bars, 25 μm; yellow size bar, 100 μm.

References

Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., Lemaigre, F.P., 2009. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325-2333.

Bhattaram, P., Penzo-Mendez, A., Sock, E., Colmenares, C., Kaneko, K.J., Vassilev, A., Depamphilis, M.L., Wegner, M., Lefebvre, V., 2010. Organogenesis relies on SoxC transcription factors for the survival of neural and mesenchymal progenitors. Nature communications 1, 9.

Carpentier, R., Suner, R.E., van Hul, N., Kopp, J.L., Beaudry, J.B., Cordi, S., Antoniou, A., Raynaud, P., Lepreux, S., Jacquemin, P., Leclercq, I.A., Sander, M., Lemaigre, F.P., 2011. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 141, 1432-1438, 1438 e1431-1434.

Christoffels, V.M., Sassi, H., Ruijter, J.M., Moorman, A.F., Grange, T., Lamers, W.H., 1999. A mechanistic model for the development and maintenance of portocentral gradients in gene expression in the liver. Hepatology 29, 1180-1192.

Clement, C.A., Ajbro, K.D., Koefoed, K., Vestergaard, M.L., Veland, I.R., Henriques de Jesus, M.P., Pedersen, L.B., Benmerah, A., Andersen, C.Y., Larsen, L.A., Christensen, S.T., 2013. TGF-beta signaling is associated with endocytosis at the pocket region of the primary cilium. Cell reports 3, 1806-1814.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., Lemaigre, F.P., 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes & development 19, 1849-1854.

Clotman, F., Libbrecht, L., Gresh, L., Yaniv, M., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2003. Hepatic artery malformations associated with a primary defect in intrahepatic bile duct development. J Hepatol 39, 686-692.

Delous, M., Yin, C., Shin, D., Ninov, N., Debrito Carten, J., Pan, L., Ma, T.P., Farber, S.A., Moens, C.B., Stainier, D.Y., 2012. Sox9b is a key regulator of pancreaticobiliary ductal system development. PLoS genetics 8, e1002754.

Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimber-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., Siveke, J.T., 2008. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48, 607-616.

Habbig, S., Bartram, M.P., Muller, R.U., Schwarz, R., Andriopoulos, N., Chen, S., Sagmuller, J.G., Hoehne, M., Burst, V., Liebau, M.C., Reinhardt, H.C., Benzing, T., Schermer, B., 2011. NPHP4, a cilia-associated protein, negatively regulates the Hippo pathway. The Journal of cell biology 193, 633-642.

Hanley, K.P., Oakley, F., Sugden, S., Wilson, D.I., Mann, D.A., Hanley, N.A., 2008. Ectopic SOX9 mediates extracellular matrix deposition characteristic of organ fibrosis. The Journal of biological chemistry 283, 14063-14071.

Jacquemin, P., Hwang, J.J., Martial, J.A., Dolle, P., Davidson, I., 1996. A novel family of developmentally regulated mammalian transcription factors containing the TEA/ATTS DNA binding domain. The Journal of biological chemistry 271, 21775-21785.

Kamachi, Y., Kondoh, H., 2013. Sox proteins: regulators of cell fate specification and differentiation. Development 140, 4129-4144.

Kellendonk, C., Opherk, C., Anlag, K., Schutz, G., Tronche, F., 2000. Hepatocyte-specific expression of Cre recombinase. Genesis 26, 151-153.

Kesavan, G., Sand, F.W., Greiner, T.U., Johansson, J.K., Kobberup, S., Wu, X., Brakebusch, C., Semb, H., 2009. Cdc42-mediated tubulogenesis controls cell specification. Cell 139, 791-801.

Kist, R., Schrewe, H., Balling, R., Scherer, G., 2002. Conditional inactivation of Sox9: a mouse model for campomelic dysplasia. Genesis 32, 121-123.

Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., Chiba, T., 2004. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127, 1775-1786.

Kuwahara, M., Yamashita, M., Shinoda, K., Tofukuji, S., Onodera, A., Shinnakasu, R., Motohashi, S., Hosokawa, H., Tumes, D., Iwamura, C., Lefebvre, V., Nakayama, T., 2012. The transcription factor Sox4 is a downstream target of signaling by the cytokine TGF-beta and suppresses T(H)2 differentiation. Nature immunology 13, 778-786.

Lefebvre, V., Dumitriu, B., Penzo-Mendez, A., Han, Y., Pallavi, B., 2007. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox)

transcription factors. The international journal of biochemistry & cell biology 39, 2195-2214.

Leitch, C.C., Lodh, S., Prieto-Echague, V., Badano, J.L., Zaghloul, N.A., 2014. Basal body proteins regulate Notch signaling via endosomal trafficking. Journal of cell science 127, 2407-2419.

Lemaigre, F.P., 2009. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 137, 62-79.

Lincoln, J., Kist, R., Scherer, G., Yutzey, K.E., 2007. Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development. Developmental biology 305, 120-132.

Manfroid, I., Ghaye, A., Naye, F., Detry, N., Palm, S., Pan, L., Ma, T.P., Huang, W., Rovira, M., Martial, J.A., Parsons, M.J., Moens, C.B., Voz, M.L., Peers, B., 2012. Zebrafish sox9b is crucial for hepatopancreatic duct development and pancreatic endocrine cell regeneration. Developmental biology 366, 268-278.

Moreno, C.S., 2010. The Sex-determining region Y-box 4 and homeobox C6 transcriptional networks in prostate cancer progression: crosstalk with the Wnt, Notch, and PI3K pathways. The American journal of pathology 176, 518-527.

Penzo-Mendez, A., Dy, P., Pallavi, B., Lefebvre, V., 2007. Generation of mice harboring a Sox4 conditional null allele. Genesis 45, 776-780.

Plumb-Rudewiez, N., Clotman, F., Strick-Marchand, H., Pierreux, C.E., Weiss, M.C., Rousseau, G.G., Lemaigre, F.P., 2004. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. Hepatology 40, 1266-1274.

Raynaud, P., Carpentier, R., Antoniou, A., Lemaigre, F.P., 2011a. Biliary differentiation and bile duct morphogenesis in development and disease. The international journal of biochemistry & cell biology 43, 245-256.

Raynaud, P., Tate, J., Callens, C., Cordi, S., Vandersmissen, P., Carpentier, R., Sempoux, C., Devuyst, O., Pierreux, C.E., Courtoy, P., Dahan, K., Delbecque, K., Lepreux, S., Pontoglio, M., Guay-Woodford, L.M., Lemaigre, F.P., 2011b. A classification of ductal plate malformations based on distinct pathogenic mechanisms of biliary dysmorphogenesis. Hepatology 53, 1959-1966.

Rockich, B.E., Hrycaj, S.M., Shih, H.P., Nagy, M.S., Ferguson, M.A., Kopp, J.L., Sander, M., Wellik, D.M., Spence, J.R., 2013. Sox9 plays multiple roles in the lung

epithelium during branching morphogenesis. Proceedings of the National Academy of Sciences of the United States of America 110, E4456-4464.

Scharer, C.D., McCabe, C.D., Ali-Seyed, M., Berger, M.F., Bulyk, M.L., Moreno, C.S., 2009. Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. Cancer research 69, 709-717.

Seymour, P.A., Freude, K.K., Tran, M.N., Mayes, E.E., Jensen, J., Kist, R., Scherer, G., Sander, M., 2007. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 104, 1865-1870.

Shih, H.P., Kopp, J.L., Sandhu, M., Dubois, C.L., Seymour, P.A., Grapin-Botton, A., Sander, M., 2012. A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. Development 139, 2488-2499.

Si-Tayeb, K., Lemaigre, F.P., Duncan, S.A., 2010. Organogenesis and development of the liver. Developmental cell 18, 175-189.

Song, S., Maru, D.M., Ajani, J.A., Chan, C.H., Honjo, S., Lin, H.K., Correa, A., Hofstetter, W.L., Davila, M., Stroehlein, J., Mishra, L., 2013. Loss of TGF-beta adaptor beta2SP activates notch signaling and SOX9 expression in esophageal adenocarcinoma. Cancer research 73, 2159-2169.

Takayama, K., Kawabata, K., Nagamoto, Y., Inamura, M., Ohashi, K., Okuno, H., Yamaguchi, T., Tashiro, K., Sakurai, F., Hayakawa, T., Okano, T., Furue, M.K., Mizuguchi, H., 2014. CCAAT/enhancer binding protein-mediated regulation of TGFbeta receptor 2 expression determines the hepatoblast fate decision. Development 141, 91-100.

Tanimizu, N., Kikkawa, Y., Mitaka, T., Miyajima, A., 2012. alpha1- and alpha5-containing laminins regulate the development of bile ducts via beta1 integrin signals. The Journal of biological chemistry 287, 28586-28597.

Tanimizu, N., Miyajima, A., 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. Journal of cell science 117, 3165-3174.

Tanimizu, N., Miyajima, A., Mostov, K.E., 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Molecular biology of the cell 18, 1472-1479.

Tchorz, J.S., Kinter, J., Muller, M., Tornillo, L., Heim, M.H., Bettler, B., 2009. Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. Hepatology 50, 871-879.

Vervoort, S.J., Lourenco, A.R., van Boxtel, R., Coffer, P.J., 2013. SOX4 mediates TGF-beta-induced expression of mesenchymal markers during mammary cell epithelial to mesenchymal transition. PloS one 8, e53238.

Walter, T.J., Sparks, E.E., Huppert, S.S., 2012. 3-dimensional resin casting and imaging of mouse portal vein or intrahepatic bile duct system. Journal of visualized experiments : JoVE, e4272.

Zhang, N., Bai, H., David, K.K., Dong, J., Zheng, Y., Cai, J., Giovannini, M., Liu, P., Anders, R.A., Pan, D., 2010. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Developmental cell 19, 27-38.

Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., Stanger, B.Z., 2009. Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727-1739.

3.2. Hepatic Notch2 deficiency leads to bile duct agenesis perinatally and secondary bile duct formation after weaning

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I performed the experiments of Fig. 3 and Fig. 6.

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Hepatic Notch2 deficiency leads to bile duct agenesis perinatally and secondary bile duct formation after weaning.

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Abstract

Notch signaling plays an acknowledged role in bile-duct development, but its involvement in cholangiocyte-fate determination remains incompletely understood. We investigated the effects of early Notch2 deletion in Notch2(fl/fl)/Alfp-Cre(tg/-) ("Notch2-cKO") and Notch2(fl/fl)/Alfp-Cre(-/-) ("control") mice. Fetal and neonatal Notch2-cKO livers were devoid of cytokeratin19 (CK19)-, Dolichos-biflorus agglutinin (DBA)-, and SOX9-positive ductal structures, demonstrating absence of prenatal cholangiocyte differentiation. Despite extensive cholestatic hepatocyte necrosis and growth retardation, mortality was only ~15%. Unexpectedly, a slow process of secondary cholangiocyte differentiation and bile-duct formation was initiated around weaning that histologically resembled the ductular reaction. Newly formed ducts varied from rare and non-connected, to multiple, disorganized tubular structures that connected to the extrahepatic bile ducts. Jaundice had disappeared in ~30% of Notch2-cKO mice by 6 months. The absence of NOTCH2 protein in postnatally differentiating cholangiocyte nuclei of Notch2-cKO mice showed that these cells had not originated from non-recombined precursor cells. Notch2 and Hnf6 mRNA levels were permanently decreased in Notch2-cKO livers. Perinatally, Foxa1, Foxa2, Hhex, Hnf1β, Cebpα and Sox9 mRNA levels were all significantly lower in Notch2-cKO than control mice, but all except Foxa2 returned to normal or increased levels after weaning, coincident with the observed secondary bile-duct formation. Interestingly, Hhex and Sox9 mRNA levels remained elevated in icteric 6 months old Notch2-cKOs, but decreased to control levels in non-icteric Notch2-cKOs, implying a key role in secondary bile-duct formation.

CONCLUSION:

Cholangiocyte differentiation becomes progressively less dependent on NOTCH2 signaling with age, suggesting that ductal-plate formation is dependent on NOTCH2, but subsequent cholangiocyte differentiation is not.

3.3. Role of liver kinase B1 (LKB1) in bile duct development

Foreword

The overall aim of this work, which was initiated in the laboratory of C. Perret (Institut Cochin, Paris) is to identify candidate genes that mediate the phenotype of *CTNNB1*-mutated hepatocellular carcinomas (HCCs). Activating mutations of the gene encoding β-catenin are found in about one third of HCCs and constitute a homogeneous subgroup of tumors with a phenotype and morphology relatively well distinct from HCC tumors without *CTNNB1* mutation. HCCs with *CTNNB1* mutations display a well differentiated phenotype characterized by microtrabecular or acinar growth pattern with acquisition of a simple-type apico-basal polarity. (Audard et al., 2007; de La Coste et al., 1998). Moreover, patients suffering from *CTNNB1*-mutated HCC have a relatively better prognosis (Monga, 2011).

The group of Prof. C. Perret selected liver kinase B1 (LKB1), encoded by the *STK11* gene, as a good candidate to mediate the effects of mutated β-catenin in HCCs. Indeed, LKB1 is overexpressed in HCCs and HCC-derived cell lines. Transcriptomic analysis of *CTNNB1*-mutated HCCs identified a LKB1 gene signature, and deletion of LKB1 in a mouse model which develops HCCs with *Ctnnb1* mutations, led to the loss of the phenotype typical of *CTNNB1*-mutated tumors (de La Coste et al., 1999). In addition, LKB1 is required for the survival of *CTNNB1*-activated hepatocytes. Together, these data suggest that LKB1 mediates at least part of the effects of *CTNNB1*-mutations in HCCs.

To better understand the role of LKB1 in the liver, phenotypic analyses of livers with specific deletion of LKB1 (*Alfp*-Cre; LKB1$^{loxp/loxp}$) were made. Liver zonation, a process dependent on functional Wnt/β-catenin signaling (Benhamouche et al., 2006) was perturbed, and expression of several β-catenin-activated target genes was reduced. Thus, these data further suggested that LKB1 is a mediator of β-catenin in normal liver (not shown).

LKB1 knockout mice were also cholestatic and this resulted from bile duct paucity. Given the expertise of our laboratory, Prof. Perret requested our collaboration to

investigate the biliary phenotype of LKB1 knockout mice. In this work, I analysed the prenatal morphogenesis of bile ducts and investigated how LKB1 controls bile duct development. My contribution will be included in a manuscript describing the interaction between LKB1, β-catenin and Notch signaling in normal liver and in HCC. The manuscript will be authored by Pierre-Alexandre Just, Alexis Poncy, Massiré Traore, Roland Teboul, Rajae Dahmani, Valérie Drouet Solenne Marmier, Hélène Gilgenkrantz, Sabine Colnot, Benoit Terris, Cédric Coulouarn, Frédéric Lemaigre and Christine Perret.

Introduction

LKB1 was originally identified as a tumor suppressor, since inactivating mutations in LKB1 in humans cause the heritable Peutz-Jeghers syndrome (Hemminki et al., 1998). LKB1 is serine/threonine kinase and forms a heterotrimeric complex that includes the LKB1/STRAD/Mo25 proteins (Boudeau et al., 2003). It is a key upstream activator of the AMP-activated protein kinase (APMK-α1 and - α2) and of 12 other AMPK-related kinases (Hardie and Alessi, 2013; Shackelford and Shaw, 2009). The LKB1-AMPK pathway negatively controls lipid biosynthesis and cell metabolism. When intracellular ATP declines, such as during nutrient deprivation or hypoxia, LKB1 activates AMPK which switches off anabolic pathways that consume ATP and NADPH, while switching on catabolic pathways that generate ATP. In the same nutritional conditions, LKB1-MAPK inhibits the mammalian target-of-rapamycin (mTOR) pathway, resulting in an arrest of cell cycle progression, inhibition of cell growth and angiogenesis (Corradetti et al., 2004; Shackelford and Shaw, 2009; Shaw et al., 2004). In addition, LKB1 promotes cell polarization. Indeed, 6 of the 14 LKB1-activated kinases, namely the MAP/microtubule affinity regulating kinase (MARK1-4) and the BR serine/threonine-protein kinase (BRSK1-2), play critical roles in regulating cell polarity (Barnes et al., 2007; Cohen et al., 2004; Kishi et al., 2005). Therefore, LKB1 is a multi-task serine/threonine kinase that controls cell metabolism, polarity and proliferation, and it is known as a tumor suppressor.

In the liver, the LKB1-AMPK pathway regulates lipid, glucose and proteins homeostasis. LKB1-deficient livers are associated with hyperglycemia with increased gluconeogenic and lipogenic gene expression (Patel et al., 2014; Shaw et al., 2005; Viollet et al., 2009). However, the role of LKB1 in liver development is poorly understood. LKB1 is required for the formation of the canalicular network, for proper localization of canalicular proteins and for hepatocyte polarization (Fu et al., 2010; Fu et al., 2011; Homolya et al., 2014; Woods et al., 2011). Moreover, Woods et al. have shown that liver-specific deletion of LKB1 in embryonic mice is associated with postnatal bile duct paucity (Woods et al., 2011).

Here, we show that deletion of LKB1 in fetal livers leads to a perturbed biliary morphogenesis and cholestasis. Gene Set Enrichement Analyses (GSEA), in which the transcriptomes of RBPJκ and LKB1 knockout livers are investigated, reveal an important cross-talk between the LKB1 and Notch signaling pathway. Therefore, LKB1 is required for normal bile duct morphogenesis and may modulate

Notch signaling, a pathway known to be essential for bile duct morphogenesis (Tchorz et al., 2009; Zong et al., 2009).

Results

LKB1 promotes maturation of bile ducts during biliary morphogenesis

To better understand the role of LKB1 in developing liver, we specifically deleted *Lkb1* using floxed *Lkb1*$^{lox/lox}$ alleles and *Alfp-Cre* (Bardeesy et al., 2002; Kellendonk et al., 2000). In the latter, Cre recombinase is active in hepatoblasts starting at E10.5. The animals were named LKBKO^{Livemb} and were born at the expected mendelian frequency. They showed severe growth retardation beginning at post-natal day 12 leading to death between day 25 and day 30 (Fig. 1B). Furthermore, the mice were cholestatic as shown by elevated serum bilirubin levels (Fig. 1C).

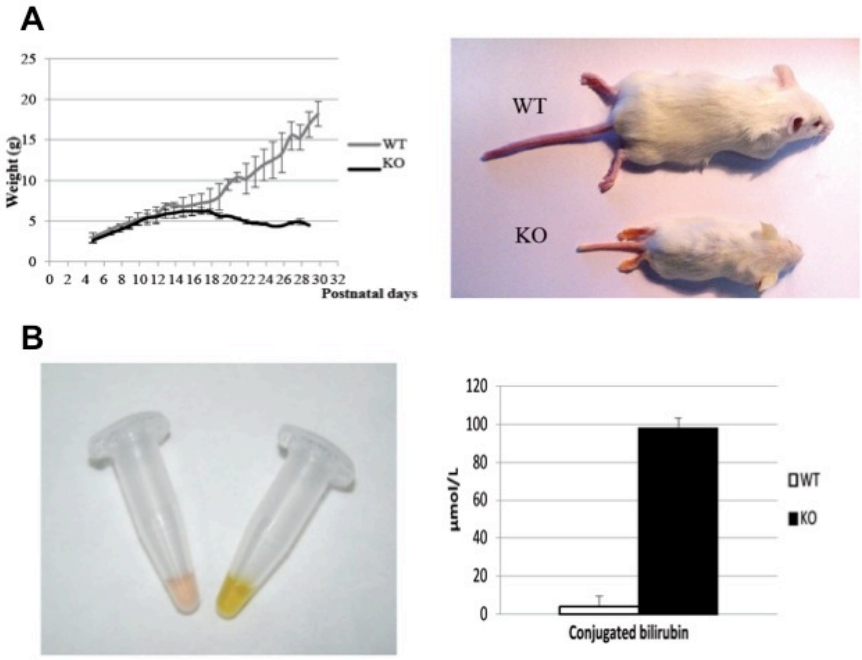

Figure 1: Phenotype of mice carrying *Lkb1* deletion in the embryonic liver. **A**: Deletion of *Lkb1* in the embryonic liver causes postnatal growth retardation beginning at day 12. Picture of WT mouse and LKBKO^{livemb} littermate at postnatal day 28d. Weight curves are from birth to post-natal day 30 in WT and LKBKO^{livemb} mice. Mice were genotyped at postnatal day 5. N=15 (control) and 8 (LKBKO^{livemb}). Error bars: standard deviations. **B**: Obstructive cholestasis in LKBKO^{livemb} mutant mice. Gross aspect of serum from a control and LKBKO^{livemb} mouse at postnatal day 19. Blood levels of conjugated bilirubin in LKBKO^{livemb} control and mutant mice at postnatal day 19. N=6 (control) and 4 (LKBKO^{livemb}). Error bars: standard deviations. (Data from P.A. Just)

To investigate the etiology of cholestasis in LKBKO^{Livemb} mice (Fig. 1C), we examined the intrahepatic bile ducts. In control livers at postnatal day 21, one to two CK19-positive bile ducts were located in each portal tract, as expected. In contrast, mutant animals failed to develop ducts but showed CK19-positive cells organized around the portal tract like in embryonic ductal plate-like structures (Fig. 2A).

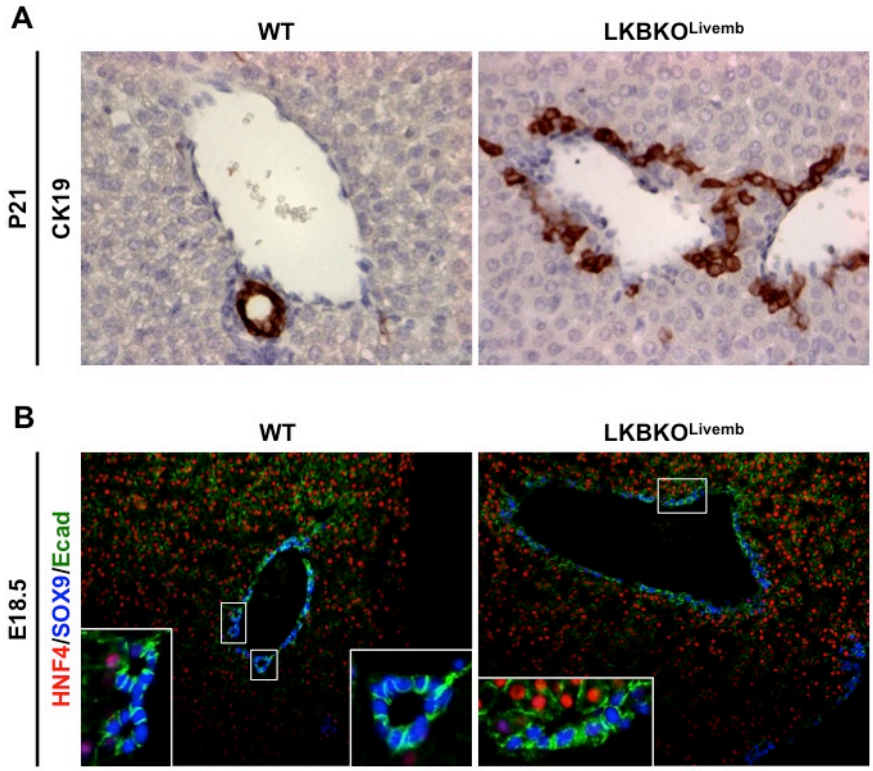

Figure 2: Lkb1 controls intrahepatic bile duct tubulogenesis. A: Lkb1 is required for intrahepatic bile ducts tubulogenesis. Representative images of 3-week old control and LKBKO^{livemb} mice liver, stained for cytokeratin 19 (cholangiocyte marker). Note the well-formed and mature bile duct in the control mouse and the ductal plate-like structure around a portal tract in mutant mouse (Data from P.A.Just). **B**: Lkb1 is required for bile duct maturation. LKBKO^{livemb} embryos were collected at E18.5 and liver sections were stained for the hepatoblast marker HNF4, and for the cholangiocyte marker SOX9. Note the symmetrical localization of SOX9 around the bile duct in control mice whereas SOX9 is only expressed in the portal layer of the asymmetric bile ducts in mutant mice.

To determine how LKB1 deletion causes defective duct formation, we analyzed bile duct morphogenesis at prenatal stages. At embryonic day (E) 18.5 developing bile ducts were radially symmetrical in control mice: all cells lining developing ducts displayed the Sox9+/HNF4- pattern. In contrast, fewer developing ducts were found in LKBKO^{Livemb} mice and all of them were still asymmetrical, *i.e.* in primitive ductal configuration. The duct lumina were surrounded on the portal side by Sox9+/HNF4- cholangiocytes and on the parenchymal side the cells were Sox9-/HNF4+ like hepatoblasts (Fig. 2B). We then tested the expression of key transcription factors of bile duct morphogenesis, namely HNF1β, HNF6 and YAP (Clotman et al., 2002; Coffinier et al., 2002; Zhang et al., 2010). These factors displayed an asymmetrical distribution in the developing ducts of mutant animals, while their expression was symmetrical in control mice (Fig 3). Similarly, the nuclear location of the Notch2 intracellular domain (N2ICD) and the expression of the Notch target gene Hes1 were asymmetrical in mutant embryos (Fig. 3). These data indicate that LKB1 is not required for cholangiocyte differentiation, since the ductal plate forms normally in LKBKO^{Livemb} mice. However, in the absence of LKB1, bile duct morphogenesis is strongly perturbed as shown by the lower number of developing ducts and their inability to mature into symmetrical and fully differentiated bile ducts at the end of gestation. Therefore LKB1 has a critical role in initiation and maturation of bile duct morphogenesis.

In addition, LKB1 is known to promote apico-basal polarization, suggesting that perturbed polarization of developing cholangiocytes could contribute to deficient morphogenesis (Cheung et al., 2012; Tanimizu et al., 2012; Tanimizu and Mitaka, 2013). However, expression and localization of several markers of biliary polarization were tested by immunolabeling experiments. These include the apical markers Osteopontin, Mucin1 and laminin. No difference was observed between wild-type mice and LKBKO^{Livemb} littermates, most likely ruling out that LKB1 impacts on morphogenesis by controling polarization (Fig. 4).

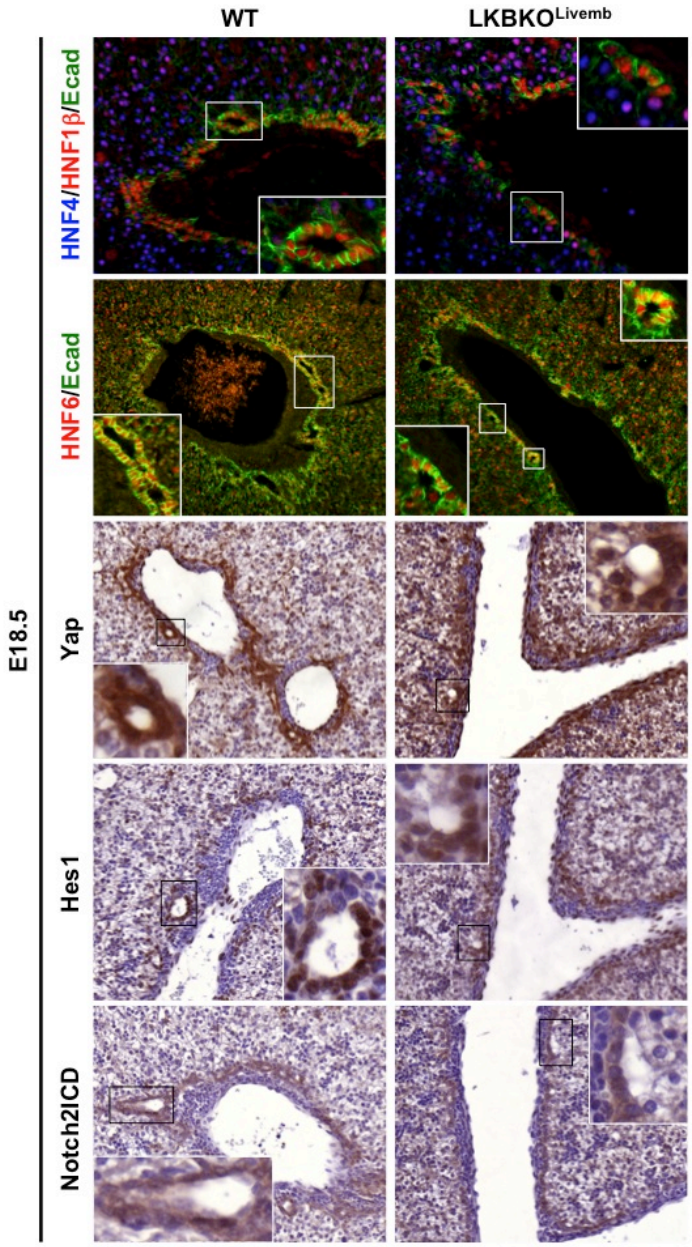

Figure 3: Expression of master regulators of bile duct development is restricted to the portal layer of primitive ductal structures in LKBKO^{livemb} mutant mice. Liver sections from E18.5 embryos were stained for HNF1β, HNF6, Yap, Hes1 and Notch2 Intracellular domain (Notch2ICD). Their expression is restricted to the portal layer of primitive ductal structures in LKBKO^{livemb} mice.

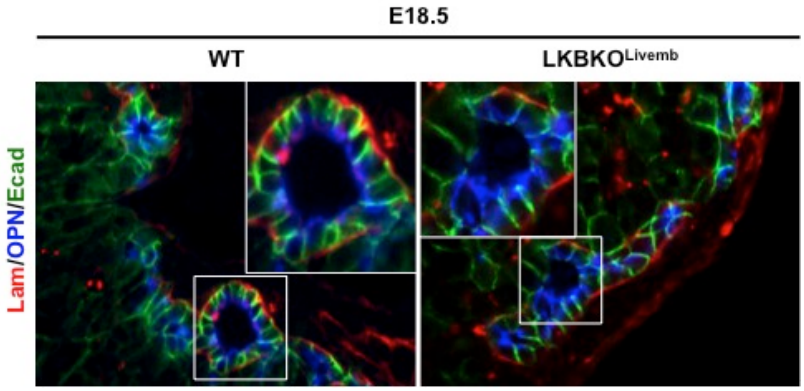

Figure 4: Expression of markers of apico-basal polarity. Liver sections from E18.5 embryos were stained for the apical marker Osteopontin (OPN), basal marker Laminin (Lam) and E-Cadherin (Ecad). Osteopontin and Laminin are normally expressed in ductal cells committed to a biliary fate.

Cross-talk between LKB1 and Notch pathways in liver (Data from P.A. Just)

The Notch pathway plays major roles in the bile duct morphogenesis, and mice with defective Notch signaling in embryonic liver display severe cholestasis resulting from perturbed biliary differentiation and morphogenesis (Hofmann et al., 2010; Kodama et al., 2004; Zong et al., 2009). The phenotype of LKBKO^{Livemb} mice is reminiscent of mice with inactivation of Hes1 or Jagged 1 (Hofmann et al., 2010; Kodama et al., 2004), implying a potential dialog between the LKB1 and Notch pathways. Supporting this hypothesis, we found that several Notch pathway targets (*Hes1*, *Hey1*, *Heyl* and *Nrarp*) were downregulated in the liver of LKBKO^{Livemb} mutant mice at post-natal days 8 and 14 (Fig. 5A). To check if LKB1 indeed cross-talks with the Notch pathway in the liver, we analyzed *Rbpj$^{lox/lox}$;Alfp-Cre* (NOTCHKO^{livemb}) mice which have a liver-specific inactivation of *RbpJk*, an essential cofactor of NICD. These mice are known to display defective biliary differentiation and cholestasis (Zong et al., 2009). Control, NOTCHKO^{livemb} and LKBKO^{Livemb} mice were sacrificed 5 days after birth and the liver transcriptomes were analyzed using microarrays. 253 non redundant genes were differentially expressed between LKBKO^{Livemb} and control mice; 237 genes were differentially expressed between NOTCHKO^{livemb} and control livers. Interestingly, a large number of genes (55 genes, *i.e.* 20-25% of each list) were common to both lists of differentially-expressed genes (Fig. 5B). Gene Set Enrichement Analysis (GSEA), revealed that the NOTCHKO^{livemb} dataset was significantly enriched in LKBKO^{livemb} signature genes (genes that were significantly down- or up-regulated), and *vice*

versa (Fig. 4C). Such data shed light on an important cross-talk between LKB1 and Notch signaling in liver.

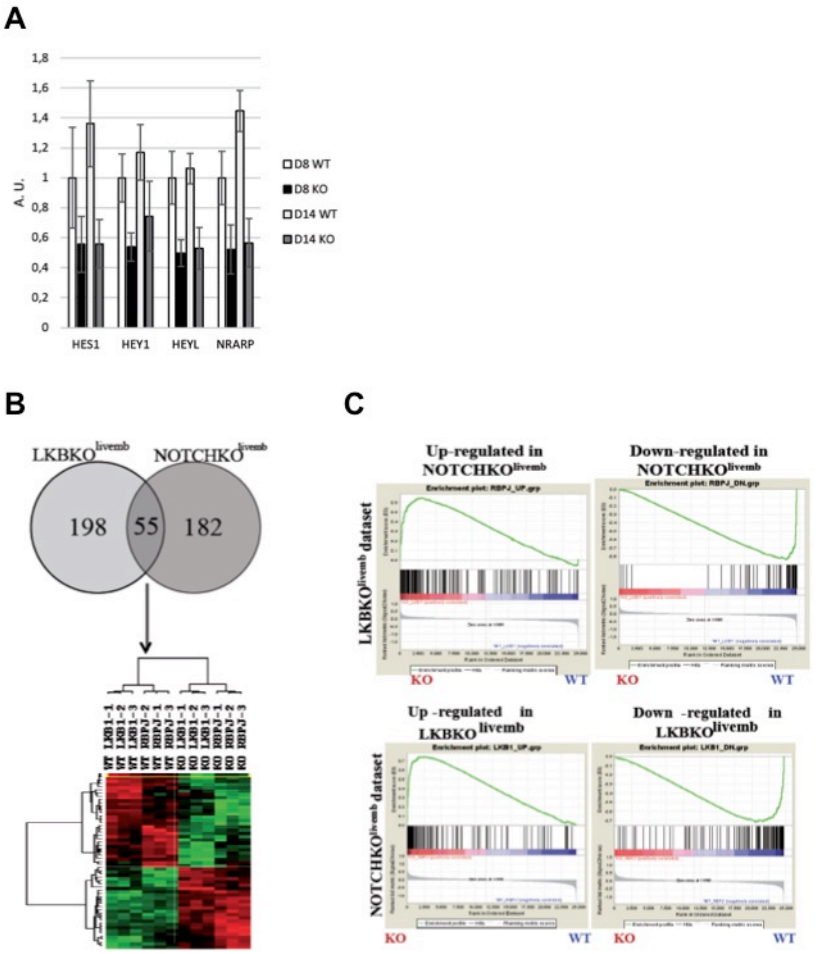

Figure 5: Livers with inactivation of *Lkb1* and Notch share common molecular features. A: Lkb1 is required for full activation of Notch signaling in the liver. RT-qPCR of Notch positive target genes (*Hey1*, *Heyl*, *Hes1* and *Nrarp*) in 8- and 14-day old control and LKBKO^{livemb} mice. N=3-4 per group. Error bars: SEM. **B**: NOTCHKO^{livemb} and LKBKO^{livemb} models share a common transcriptomic signature. Top: Venn diagram of differentially expressed genes ($p<0.05$, fold change>1.5) on micro-array transcriptomic chips comparing LKBKO^{livemb} and control mice (5-day old) models. Bottom: supervised clustering of the 55-gene common signature of control and mutant mice of both models. **C**: The Lkb1 transcriptomic signature is enriched in the NOTCHKO^{livemb} model and *vice versa*. Gene Set Enrichement Analysis using the different datasets. All gene sets were significantly enriched at nominal p-value <1%.

Discussion

Mice with liver-specific inactivation of LKB1 (LKBKO^{Livemb}) show bile duct paucity as a result of biliary dysmorphogenesis. LKB1 was not required for cholangiocyte differentiation as ductal plate formed correctly in LKBKO^{Livemb} mice. In contrast, LKB1 was essential for primitive duct formation and bile duct maturation.

Several master regulators of bile duct development were investigated. At the end of gestation, all tested factors known to be critical for biliary morphogenesis (HNF6, HNF1β and Yap) displayed asymmetrical expression, like in primitive ductal structures. The perturbed expression of these transcription factors could contribute to the biliary phenotype (Clotman et al., 2002; Coffinier et al., 2002; Yimlamai et al., 2014). However, we cannot eliminate the possibility that their asymmetrical expression simply reflects bile duct immaturity, in which case abnormal expression of the factors would not cause but be a consequence of the phenotype.

Similarly, the nuclear location of the Notch intracellular domain (NICD) and the expression of the Notch target gene Hes1 were asymmetrical in ductal structures of mutant embryos. As for HNF6, HNF1β and Yap, we cannot conclude if the abnormal expression profile of Notch is a cause or a consequence of biliary immaturity. However, it has been shown that LKB1 modulates Notch signaling during differentiation of secretory cell lineages within the intestine (Shorning et al., 2009). In the present work, we found a cross-talk between the LKB1 and Notch pathways in the liver. However, how this crosstalk contributes to bile duct morphogenesis is not yet clear. Analysis of the genes that are common targets of the Notch and LKB1 pathways did not reveal obvious candidate regulators of duct morphogenesis. Importantly, the transcriptome analysis was performed at postnatal day 5, when biliary morphogenesis is already achieved. This suggests that genes essential for bile duct morphogenesis may have been overlooked in the transcriptome analysis, and prompts the need for refined gene expression analysis during development.

References

Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., Lemaigre, F.P., 2009. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325-2333.

Audard, V., Grimber, G., Elie, C., Radenen, B., Audebourg, A., Letourneur, F., Soubrane, O., Vacher-Lavenu, M.C., Perret, C., Cavard, C., Terris, B., 2007. Cholestasis is a marker for hepatocellular carcinomas displaying beta-catenin mutations. The Journal of pathology 212, 345-352.

Bardeesy, N., Sinha, M., Hezel, A.F., Signoretti, S., Hathaway, N.A., Sharpless, N.E., Loda, M., Carrasco, D.R., DePinho, R.A., 2002. Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. Nature 419, 162-167.

Barnes, A.P., Lilley, B.N., Pan, Y.A., Plummer, L.J., Powell, A.W., Raines, A.N., Sanes, J.R., Polleux, F., 2007. LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. Cell 129, 549-563.

Benhamouche, S., Decaens, T., Godard, C., Chambrey, R., Rickman, D.S., Moinard, C., Vasseur-Cognet, M., Kuo, C.J., Kahn, A., Perret, C., Colnot, S., 2006. Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. Developmental cell 10, 759-770.

Boudeau, J., Baas, A.F., Deak, M., Morrice, N.A., Kieloch, A., Schutkowski, M., Prescott, A.R., Clevers, H.C., Alessi, D.R., 2003. MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. The EMBO journal 22, 5102-5114.

Cheung, I.D., Bagnat, M., Ma, T.P., Datta, A., Evason, K., Moore, J.C., Lawson, N.D., Mostov, K.E., Moens, C.B., Stainier, D.Y., 2012. Regulation of intrahepatic biliary duct morphogenesis by Claudin 15-like b. Developmental biology 361, 68-78.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. Development 129, 1819-1828.

Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., Barra, J., 2002. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129, 1829-1838.

Cohen, D., Brennwald, P.J., Rodriguez-Boulan, E., Musch, A., 2004. Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton. The Journal of cell biology 164, 717-727.

Corradetti, M.N., Inoki, K., Bardeesy, N., DePinho, R.A., Guan, K.L., 2004. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes & development 18, 1533-1538.

de La Coste, A., Mignon, A., Fabre, M., Gilbert, E., Porteu, A., Van Dyke, T., Kahn, A., Perret, C., 1999. Paradoxical inhibition of c-myc-induced carcinogenesis by Bcl-2 in transgenic mice. Cancer research 59, 5017-5022.

de La Coste, A., Romagnolo, B., Billuart, P., Renard, C.A., Buendia, M.A., Soubrane, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A., Perret, C., 1998. Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proceedings of the National Academy of Sciences of the United States of America 95, 8847-8851.

Fu, D., Wakabayashi, Y., Ido, Y., Lippincott-Schwartz, J., Arias, I.M., 2010. Regulation of bile canalicular network formation and maintenance by AMP-activated protein kinase and LKB1. Journal of cell science 123, 3294-3302.

Fu, D., Wakabayashi, Y., Lippincott-Schwartz, J., Arias, I.M., 2011. Bile acid stimulates hepatocyte polarization through a cAMP-Epac-MEK-LKB1-AMPK pathway. Proceedings of the National Academy of Sciences of the United States of America 108, 1403-1408.

Hardie, D.G., Alessi, D.R., 2013. LKB1 and AMPK and the cancer-metabolism link - ten years after. BMC biology 11, 36.

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Hoglund, P., Jarvinen, H., Kristo, P., Pelin, K., Ridanpaa, M., Salovaara, R., Toro, T., Bodmer, W., Olschwang, S., Olsen, A.S., Stratton, M.R., de la Chapelle, A., Aaltonen, L.A., 1998. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 391, 184-187.

Hofmann, J.J., Zovein, A.C., Koh, H., Radtke, F., Weinmaster, G., Iruela-Arispe, M.L., 2010. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 137, 4061-4072.

Homolya, L., Fu, D., Sengupta, P., Jarnik, M., Gillet, J.P., Vitale-Cross, L., Gutkind, J.S., Lippincott-Schwartz, J., Arias, I.M., 2014. LKB1/AMPK and PKA control ABCB11 trafficking and polarization in hepatocytes. PloS one 9, e91921.

Kellendonk, C., Opherk, C., Anlag, K., Schutz, G., Tronche, F., 2000. Hepatocyte-specific expression of Cre recombinase. Genesis 26, 151-153.

Kishi, M., Pan, Y.A., Crump, J.G., Sanes, J.R., 2005. Mammalian SAD kinases are required for neuronal polarization. Science 307, 929-932.

Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., Chiba, T., 2004. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 127, 1775-1786.

Monga, S.P., 2011. Role of Wnt/beta-catenin signaling in liver metabolism and cancer. The international journal of biochemistry & cell biology 43, 1021-1029.

Patel, K., Foretz, M., Marion, A., Campbell, D.G., Gourlay, R., Boudaba, N., Tournier, E., Titchenell, P., Peggie, M., Deak, M., Wan, M., Kaestner, K.H., Goransson, O., Viollet, B., Gray, N.S., Birnbaum, M.J., Sutherland, C., Sakamoto, K., 2014. The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver. Nature communications 5, 4535.

Raynaud, P., Carpentier, R., Antoniou, A., Lemaigre, F.P., 2011. Biliary differentiation and bile duct morphogenesis in development and disease. The international journal of biochemistry & cell biology 43, 245-256.

Shackelford, D.B., Shaw, R.J., 2009. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nature reviews. Cancer 9, 563-575.

Shaw, R.J., Bardeesy, N., Manning, B.D., Lopez, L., Kosmatka, M., DePinho, R.A., Cantley, L.C., 2004. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer cell 6, 91-99.

Shaw, R.J., Lamia, K.A., Vasquez, D., Koo, S.H., Bardeesy, N., Depinho, R.A., Montminy, M., Cantley, L.C., 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 310, 1642-1646.

Shorning, B.Y., Zabkiewicz, J., McCarthy, A., Pearson, H.B., Winton, D.J., Sansom, O.J., Ashworth, A., Clarke, A.R., 2009. Lkb1 deficiency alters goblet and paneth cell differentiation in the small intestine. PloS one 4, e4264.

Tanimizu, N., Kikkawa, Y., Mitaka, T., Miyajima, A., 2012. alpha1- and alpha5-containing laminins regulate the development of bile ducts via beta1 integrin signals. The Journal of biological chemistry 287, 28586-28597.

Tanimizu, N., Mitaka, T., 2013. Role of grainyhead-like 2 in the formation of functional tight junctions. Tissue barriers 1, e23495.

Tchorz, J.S., Kinter, J., Muller, M., Tornillo, L., Heim, M.H., Bettler, B., 2009. Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. Hepatology 50, 871-879.

Viollet, B., Guigas, B., Leclerc, J., Hebrard, S., Lantier, L., Mounier, R., Andreelli, F., Foretz, M., 2009. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta physiologica 196, 81-98.

Woods, A., Heslegrave, A.J., Muckett, P.J., Levene, A.P., Clements, M., Mobberley, M., Ryder, T.A., Abu-Hayyeh, S., Williamson, C., Goldin, R.D., Ashworth, A., Withers, D.J., Carling, D., 2011. LKB1 is required for hepatic bile acid transport and canalicular membrane integrity in mice. The Biochemical journal 434, 49-60.

Yimlamai, D., Christodoulou, C., Galli, G.G., Yanger, K., Pepe-Mooney, B., Gurung, B., Shrestha, K., Cahan, P., Stanger, B.Z., Camargo, F.D., 2014. Hippo pathway activity influences liver cell fate. Cell 157, 1324-1338.

Zhang, N., Bai, H., David, K.K., Dong, J., Zheng, Y., Cai, J., Giovannini, M., Liu, P., Anders, R.A., Pan, D., 2010. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Developmental cell 19, 27-38.

Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., Stanger, B.Z., 2009. Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727-1739.

4. GENERAL DISCUSSION

This thesis sheds light on several aspects of bile duct development. First, SOX4 and TEAD2 were identified as new specific markers of developing ducts. Second, SOX4 was identified as a new regulator of bile duct development which cooperates with SOX9, another member of the SOX family. Third, in collaboration with the laboratory of Prof. W. Lamers we demonstrated that, contrary to current belief, Notch2 can be dispensable for postnatal bile duct regeneration. Fourth, in collaboration with Prof. C. Perret, we showed that Notch potentially cooperates with LKB1 which we showed to be essential for bile duct maturation during development.

Mechanism of action of SOX4 and SOX9 in bile duct development:* in vivo *analysis

We have shown that SOX4, SOX9 and LKB1 are essential for bile duct development. However, the mechanisms of action of these proteins during biliary developmental is only partially understood.

When investigating the function of SOX factors, we paid particular attention to their ability to control the expression of mediators of the TGFβ, Notch and Hippo/Yap signaling. Indeed, these signaling pathways drive biliary differentiation and morphogenesis and they are modulated by SOX4 and SOX9 in other developmental processes and in tumorigenesis (Bhattaram et al., 2010; Delous et al., 2012; Kadaja et al., 2014; Moreno, 2010; Scharer et al., 2009; Seymour et al., 2007; Shih et al., 2012; Song et al., 2014; Vervoort et al., 2013; Yimlamai et al., 2014). Therefore, abnormal expression of mediators, target genes or co-activators of these pathways in the absence of SOX4 and SOX9 suggests that these factors control bile duct development by modulating TGFb, Notch and Hippo/Yap signaling.

With cholangiocyte differentiation, apico-basal polarity is also strongly perturbed in the absence of SOX4 and/or SOX9. Epithelial apico-basal polarity is essential for the directional transport of ions and water in cholangiocytes and for the formation and/or maintenance of the luminal structure. Moreover, tubulogenesis via correct apico-basal polarity could impact on cell differentiation (Kesavan et al., 2009).

Importantly, laminin deposition around developing ducts is affected in our knockout mice. SOX4 and SOX9 control extracellular matrix organization and laminin deposition in developmental process (Lincoln et al., 2007; Paul et al., 2014; Rockich et al., 2013). Moreover, epithelial cells receive signals form ECM, and more particularly from laminin, to establish and to maintain apico-basal polarity (Miner and Yurchenco, 2004). This is notably the case during development of bile ducts where the laminin/β1-integrin signaling is essential for the formation and maintenance of apico-basal polarity of cholangiocytes (Tanimizu et al., 2012; Tanimizu et al., 2007). Therefore, control of laminin expression by SOX4 and SOX9 represents a potential mechanism to explain defects in apico-basal polarity in the absence of SOX4 and SOX9.

In figure 13 we propose an updated version of the gene regulatory network driving biliary differentiation; this figure includes established knowledge by others and our data on the role of SOX factors.

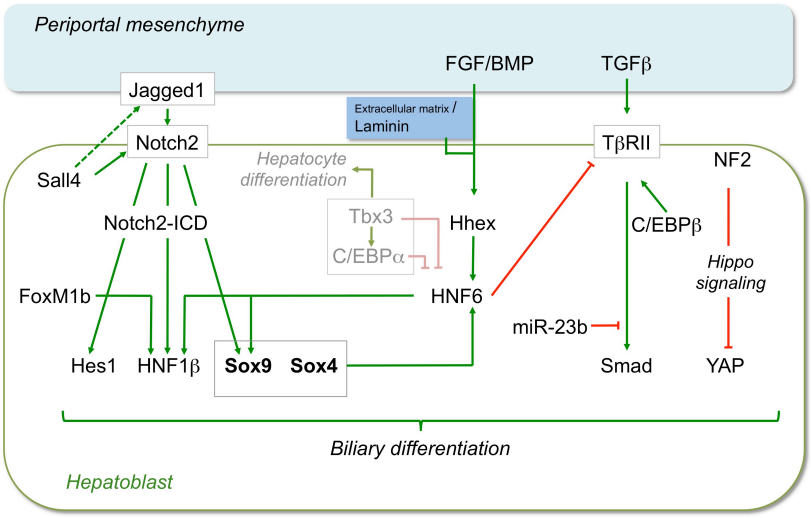

Figure 13. Schematic overview of gene interactions operating during differentiation of hepatoblasts to cholangiocytes.

Mechanism of action of SOX4 and SOX9 in bile duct development:* in vitro *analysis

We attempted to identify a cross-talk between SOX factors and the signaling pathways involved in bile duct development by using an *in vitro* model of cultured hepatoblasts. It has been shown by our group and by others that cultured hepatoblasts can differentiate toward the biliary lineage when the TGFβ, Notch and Hippo/Yap pathways are activated. Indeed, treating hepatoblasts with TGFβ or infecting them with a retrovirus coding for the constitutively active form of Notch (NICD) or Yap (unpublished data) induces biliary gene expression and represses hepatocyte gene expression (Antoniou et al., 2009; Tanimizu and Miyajima, 2004). Moreover, cultured hepatoblasts in three-dimensional embedded gel containing laminin (like Matrigel) generate cystic and polarized structures (Tanimizu et al., 2007).

Therefore, we developed an *in vitro* model of cultured hepatoblast lines where SOX4 and SOX9 expression was downregulated. In these experiments we attempted to differentiate the cells *in vitro* toward the biliary lineage or to generate three-dimensional ductular structures, and verified if the SOX-deficient cells responded like wild-type cells to activation of the TGFβ, Notch and Hippo/Yap signaling or to the presence of laminin (Matrigel) in culture.

The cultured hepatoblasts in which SOX4 and SOX9 expression was downregulated, differed from the model presented in chapter 3.1. (primary culture of purified hepatoblasts) and were instead derived from *Sox4*$^{loxP/loxP}$ livers. SOX4 was inactivated by expressing Cre recombinase (adenoviral Cre expression), and SOX9 expression was reduced by a shRNA approach to generate cells knockout for SOX4 and knockdown (50%) for SOX9. The cultured hepatoblast lines were obtained according to the protocol designed by Strick-Marchand et al. (Strick-Marchand and Weiss, 2002).

These experiments did not generate conclusive data, mainly because we were unable to use the cells (wild-type and SOX-deficient) to faithfully recapitulate biliary differentiation and bile duct moprhogenesis *in vitro*. Moreover, our unability to fully knock down SOX9 may have hampered the capacity of our in vitro hepatoblasts model to recapitulate the in vivo phenotype (more details below).

Treating the cells with TGFb induced a subset of biliary genes, like in earlier experiments (Antoniou et al., 2009); this was observed with wild-type and SOX-factor-deficient hepatoblasts, suggesting that the SOX factors do not control TGFb-induced expression of biliary markers. We did not manage to efficiently stimulate

Notch signaling in this *in vitro* model. However, downregulation of SOX4 and SOX9 did not perturb endogenous expression of components or target genes of the Notch signaling, suggesting that SOX factors do not control Notch signaling in hepatoblasts. The retroviral vector-mediated overexpression of a constitutively active form of Yap in cultured hepatoblasts strongly increased biliary gene expression. This effect was also observed in SOX4-deficient hepatoblasts even though expression of the Yap-coactivator Tead2 was drastically downregulated. This result suggests that the Hippo/Yap signaling controls biliary differentiation independently of the SOX4-Tead2 cascade. However, the other Tead factors were well expressed in our cultured hepatoblasts as compared to their weak expression level in fetal livers, raising the possibility that in these experiments, they compensated for the reduced levels of Tead2. Moreover, laminin expression was not perturbed in our SOX-factor-deficient hepatoblasts suggesting that SOX factors do not control laminin expression. However, our 2-dimensional culture model did not enable us to verify if SOX factors impact on laminin deposition. Importantly, in our *in vitro* model of cultured hepatoblasts, we totally inactivated SOX4 expression but SOX9 expression was only reduced by approximately 50%. Along with the fact that *Sox4*$^{loxp/loxp}$*; Sox9*$^{loxp/+}$*; AlfpCre* mice only showed a slight and transient phenotype, the residual expression of SOX9 could be sufficient to ensure biliary differentiation in cultured hepatoblasts. This remark must be taken in mind when assessing our data on FGF and Wnt signaling (see below).

Moreover, in three-dimensional culture, hepatoblasts can form tubes but this does not occur according to a process that includes transient asymmetry, like bile duct development *in vivo*. Therefore the 3D culture model is not appropriate to test the function of signaling pathways –and their control by SOX factors- during the morphogenic step.

Signaling pathways and transcription factors form an interconnected and dynamic network that robustly control bile duct development. Indeed, specific activation of one signaling pathway in cultured hepatoblasts induces activation of the other and can promote expression of biliary genes. Indeed, treating cultured hepatoblasts with TGFb or overexpression of Yap promoted biliary differentiation, but also induced TGFb, Notch, Hippo/Yap and laminin activation while chemical inhibition of TGFb and Notch pathways induced hepatocyte differentiation and inhibited these signaling pathways (data not shown). Work by others showed that overexpression of Yap in adult hepatocytes induces expression of biliary-enriched transcription factors (SOX9, HNF1b, HNF6), deposition of extracellular matrix and activation of signaling pathways (Notch and TGFb) (Yimlamai et al., 2014). Therefore, although

our data show that mediators of several signaling pathways are affected by the absence of SOX4 and SOX9, our results do not eliminate the possibility that SOX factors actually regulate a more limited number of signaling pathways whose perturbation subsequently affect the function of others.

Wnt and FGF signaling are also involved in biliary development (see § 1.3.4. Signaling pathways controlling bile duct development), but their connection with SOX4 and SOX9 remains unexplored. In pancreas, a SOX9/FGF10/FGFR2 feed-forward loop has been shown to avoid that pancreatic progenitor cells commit to a hepatocyte/hepatoblast cell fate (Seymour et al., 2012). Therefore, a similar mechanism operating in liver may maintain commitment of biliary cells and prevent them from differentiating to hepatocytes. This would be in line with our observation that duct-lining cells in the absence of SOX4 and SOX9 express hepatocyte genes. However, we found no evidence for perturbed FGF signaling in our *in vivo* and *in vitro* experiments with loss-of-function of SOX4 and SOX9. Immunolabeling and RT-qPCR experiments showed no difference in the expression of FGFR1 and FGFR2 between WT and knockout mice. Moreover, no effect on biliary gene expression were observed after treating the cultured hepatoblasts with FGF2 or FGF10. However, considering the large number of FGF ligands and receptors, it is possible that SOX4 and SOX9 impact on FGF signaling during biliary development through additional receptors and ligands than those involved during biliary development in chick or during pancreas development (Seymour et al., 2012; Yanai et al., 2008).

Along the same lines, SOX4 and SOX9 interact with Wnt/b-catenin signaling in several biological processes but there is no evidence for such interaction during bile duct development (Bhattaram et al., 2014; Wang et al., 2013). Immunolabeling of b-catenin showed no difference between WT and knockout mice. However, we were not able to detect nuclear b-catenin. Moreover, treating the cultured hepatoblasts with Wnt induced biliary gene expression to a similar level in wild-type and SOX-factor-deficient cells, suggesting that the SOX factors do not control Wnt-induced expression of biliary markers.

MicroARNs represent an additional mechanism for controlling bile duct development (Hand et al., 2009; Rogler et al., 2009). Since SOX4 directly regulates Dicer, Argonaute 1 and RNA helicase A – three components of the RNA-induced silencing complex (RISC) – in melanoma and prostate cancer, and since SOX9 regulates microRNAs expression during testis differentiation, a regulation of microRNA expression by SOX4 and SOX9 could represent an additional control of bile duct development. This hypothesis may be addressed by performing

microarray or RNA-seq experiments on SOX factor-deficient cells, while keeping in mind the caveats noted above regarding the use of cultured cells.

Secondary bile-duct formation occurs after weaning when first bile duct development has been impaired

In mice when Notch2 has been deleted early (*Alfp*Cre; *Notch2*$^{loxp/loxp}$, Notch2-cKO), no biliary cells are observed at the end of gestation and livers are devoid of bile ducts during the first weeks of life, leading to cholestasis in all knockout mice. However, around birth, some SOX9-positive cells appear at the vicinity of portal veins and their number increases with time until forming, after weaning, dilated and distorted bile ducts around larger portal veins; this occured in 30% of Notch2-cKOs. In addition, the latter are no longer cholestatic since the newly formed bile ducts constitute a dilated and truncated intrahepatic biliary tree connected to the extrahepatic biliary tract.

These observations revealed that secondary bile duct formation occurs in these mice (Falix et al., 2014). A similar phenomenon is observed in Yap knockout mice, in RBPJk/HNF6 double-knockout mice and in our Sox4/Sox9ko mice: newborn mice show little or no biliary cell or only poorly developed ductal-like structures, but after several weeks a number of dilated bile ducts appear near the hilum (Walter et al., 2014; Zhang et al., 2010). Therefore, it seems that secondary bile duct formation can occur after weaning when normal bile duct development has been severly impaired. What is the origin of these biliary cells and how do they develop into connected bile ducts? These newly formed bile ducts are enlarged and distorted but they are lined by cells expressing biliary markers and exhibiting correct apico-basal polarity (data not shown). The absence of transcription factors (SOX4, SOX9 and HNF6) or active signaling pathways (Notch and Hippo/Yap) essential for differentiation and polarization of cholangiocytes and for biliary morphogenesis during embryogenesis does not inhibit secondary postnatal bile-duct formation. In damaged liver, hepatocytes can transdifferentiate into biliary cells and can be incorporated into newly formed bile ducts (Sekiya and Suzuki, 2014; Yanger et al., 2013). Therefore, in the absence of cholangiocytes, transdifferentiation of hepatocytes into biliary cells seems to be the most likey way to allow secondary bile duct formation. This process is slower than embryonic bile duct formation and may require additional extracellular and intracellular signaling pathways. Hepatocyte growth factor (HGF) and epidermal growth factor (EGF)

signaling, both mediated by phosphatidylinositol 3-kinase (PI3K) (Limaye et al., 2008) are good candidates.

Phenotypic differences depending on the choice of the Cre-recombinase

In the article of Falix et al., we showed that the biliary phenotype of conditional deletion of *Notch2* depends on the selected Cre-recombinase. Indeed, liver-specific deletion of *Notch2* using the *Alfp*-Cre impaired hepatoblasts commitment into biliary fate as shown by the absence of ductal plate cells in embryonic liver. This led to a total absence of intrahepatic bile ducts during the first potsnatal weeks of the Notch2 knockout mice (Falix et al., 2014). These results differ from those obtained when Notch2 is deleted using *Albumin*-Cre. In the latter, no difference was observed in the formation of the ductal plate as compared to the wild type animals. However, *Alb*-Cre; *Notch2*$^{loxp/loxp}$ mice suffered from bile duct paucity after birth (Geisler et al., 2008; Lozier et al., 2008). The discrepancies between these studies result from the properties Cre-recombinase-coding transgenes. Indeed, excision of *Notch2* using *Alfp*-Cre mice occurs approximately 4 days earlier than using *Alb*-Cre (see discussion in 3.2. Hepatic Notch2 deficiency leads to bile duct agenesis perinatally and secondary bile duct formation after weaning). In other words, using *Alfp*-Cre, *Notch2* is deleted early enough to impair biliary differentiation and consequently block the formation of the ductal plate (between E11.5 and E15.5). Instead, when using *Alb*-Cre, *Notch2* deletion occurs after biliary commitment resulting in a normal ductal plate formation but in impaired morphogenesis of bile ducts. A similar observation is made when analyzing the biliary phenotype of *Foxa3*-Cre; *RBPJk*$^{loxp/loxp}$ mice and *Alfp*-Cre; *RBPJk*$^{loxp/loxp}$ mice (Zong et al., 2009). The *Foxa3*-Cre mouse strain allows recombination earlier than the *Alfp*-Cre strain. Therefore, *RBPJk* deletion leads to impaired ductal plate formation only when *Foxa3*-Cre is used (Zong et al., 2009). Surprisingly, whereas both *Alfp*-Cre-mediated deletion of *Notch2* or *RBPJk* disrupts Notch signaling during bile duct development, only *Notch2* deletion disrupts hepatoblasts commitment into biliary fate. The phenotypic differences between the two conditional knockouts could be do to a greater stability of the mRNA or protein of RBPJk which could delay the effect of *RBPJk* excision by *Alfp*-Cre as compared to *Notch2*.

These results illustrate the importance of the choice of the Cre-recombinase when studying developmental process. The specificity, the timing and the efficiency of the Cre-recombinase are primordial elements to take into account. Indeed, a

precocious gene deletion could affect the study of the role of this gene on later developmental process (i.e. *Alfp*-Cre-mediated *Notch2* deletion impacts on biliary commitment and thus is useless to study bile duct morphogenesis contrary to the *Alb*-Cre-mediated *Notch2* deletion). Similarly, a belated gene deletion will never be useful to investigate the role of a gene in an earlier developmental process. In this thesis, we used *Alfp*-Cre-mediated gene deletion in order to study the role of SOX4, SOX9, Notch2 and LKB1 during bile duct development. In our knowledge, this Cre-recombinase offers the best alternative to study bile duct development because its specificity, it targets only hepatoblasts; because its efficiency, almost all hepatoblasts are targeted; and because its timing, the Cre-recombinase is express since E10.5 (Kellendonk et al., 2000) and gene deletion occurs soon enough to study the role of these genes on biliary commitment (SOX9 protein is undetectable since E11.5 (Antoniou et al., 2009) and *Sox4* mRNA has disappeared before E13.5 (see 3.1. Fig. S1A), no data for Notch2 and LKB1).

A possible role of SOX4 and SOX9 in liver regeneration and cancer

The liver has a remarkable capacity to regenerate. Two mechanisms are responsible for restoring liver mass during regeneration. The first one rests on proliferation and self-renewal of hepatocytes while the second consists in activation of dormant liver progenitor cells (LPC) into transit-amplifying cells (oval cells) which proliferate (ductular reaction) and differentiate into hepatocytes or biliary cells (Duncan et al., 2009; Yanger and Stanger, 2011). The contribution of LPC to the pool of regenerated hepatocytes is still highly debated. Some studies report that LPC minimally contribute to liver regeneration while other studies reveal that LPC are a major source of newly-formed hepatocytes. The severity of liver damage influences the extent of LPC-driven liver regeneration (Carpentier et al., 2011; Espanol-Suner et al., 2012; Furuyama et al., 2011; Malato et al., 2011). Indeed, the contribution of LPCs to hepatocyte regeneration is marginal after moderate liver injury, while it becomes essential after extreme hepatocyte loss (Choi et al., 2014; He et al., 2014). Importantly, LPCs reside in the vicinity of the portal vein, probably in canals of Hering, and at least a fraction of them derives from biliary lineage, namely from the ductal plate (Carpentier et al., 2011). Therefore, liver regeneration is strongly impaired when bile duct development has been formerly disrupted (He et al., 2014). This raises questions about the role of SOX4 and SOX9 in biliary differentiation: are LPCs present and functional in *Sox4* and/or *Sox9* deficient livers? Preliminary experiments do not show significant differences in the regenerative capacity of WT, Sox4ko and Sox9ko livers after choline-deficient

ethionine-supplemented (CDE) and 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) diet-induced liver injury (data from R. Carpentier; not shown). However, in *Sox4* and *Sox9* single knockout mice, defects in biliary differentiation are transient and therefore unlikely to impact on LPC formation and function. Therefore, a similar experiment with double Sox4/Sox9ko mice should be considered in order to investigate the cooperative role of SOX4 and SOX9 in liver regeneration.

Biliary repair is a fundamental aspect during liver regeneration after partial hepatectomy or liver injury. Indeed, failure to regenerate a properly branched intrahepatic biliary network results in parenchymal necrosis and fibrosis. Notch signaling appears to be primordial in this process. In the periportal area, Jagged1-expressing myofibroblasts activate Notch signaling in LPCs and this results in commitment of the latter to the biliary lineage (Boulter et al., 2012). Moreover, Notch signaling is essential for tubular morphogenesis during liver regeneration (Fiorotto et al., 2013). Alternatively, during liver injury, some hepatocytes transdifferentiate in a Notch-dependent way into biliary cells and participate to the biliary repair (Jeliazkova et al., 2013; Yanger et al., 2013). Given that SOX4 and SOX9 modulate Notch signaling during bile duct development, we speculate that they are also required during Notch-dependent biliary repair.

SOX4 and SOX9 are overexpressed in a subset of HCC and CCA. However, their role in liver cancer is poorly investigated (see § 1.4.2. Developmental and tumorigenic functions of SOX4 and SOX9). Conversely, the role of Notch and Yap signaling has been widely investigated in HCC and CCA. Approximately, 33% and 50% of human HCCs show a Notch gene signature or aberrant overexpression and nuclear expression of Yap, respectively (Villanueva et al., 2012; Zhao et al., 2007). Moreover, constitutive activation of Notch and Yap signaling in mouse liver leads to HCC and CCA development (Avruch et al., 2011; Geisler and Strazzabosco, 2014). These oncogenic pathways are linked in liver cancer since Yap regulates Jagged 1 and Notch2 expression and activates Notch signaling in human HCC (Tschaharganeh et al., 2013; Yimlamai et al., 2014). Consistent with the fact that SOX9 is a direct target of Notch and Yap signaling in liver, Notch- and Yap-induced HCC show high SOX9 expression (Dill et al., 2013; Villanueva et al., 2012; Yimlamai et al., 2014; Zong et al., 2009). Moreover, SOX9 expression is more prevalent in HCCs with Notch signature than in HCC without such signature (Villanueva et al., 2012). In addition, SOX4 expression is highly up-regulated in Yap-induced HCC (Dong et al., 2007; Liu-Chittenden et al., 2012). These data suggest that SOX4 and SOX9 have an active role in Notch- and Yap-induced liver cancer (Capaccione et al., 2014; Moreno, 2010; Scharer et al., 2009; Song et al.,

2014). Therefore, investigating the role of SOX4 and SOX9 in liver cancer will improve our knowledge on tumorigenic mechanisms.

In vitro generation of cholangiocytes

Cholangiocytes have important physiological functions such as bile elimination, and regulation of bile composition. Moreover, humans may suffer from several cholangiopathies; they represent the main indications for liver transplantation in pediatrics (Strazzabosco et al., 2005). In addition, LPCs that are activated in injured liver belong to the cholangiocyte lineage. For these reasons, the availability of *bona fide* cholangiocytes *in vitro* is essential for understanding pathophysiology of biliary diseases and liver regeneration. These cells could be also useful for therapeutic strategies, including cell replacement therapies and constructing liver tissues by bioengineering. However, the limited number of cholangiocytes (3-5% of the total liver mass) and their low proliferating capacity have considerably restricted the development of *in vitro* cell models. At the present time, cholangiocytes cultured in vitro derive from cholangiocarcinoma or correspond to cholangiocytes immortalized by SV40 large T antigen, and cannot be considered equivalent to normal cholangiocytes.

A first strategy to obtain in vitro cholangiocytes is to differentiate embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) toward the cholangiocyte lineage by using growth factors. Using this method, Dianat *et al*. have successfully obtained cholangiocyte-like cells which express biliary markers (SOX9, OPN, CK19, HNF1b, Cystic fibrosis transmembrane conductance regulator (CFTR)), form branching tubular structures in 3D culture system and transport fluorescent bile salt (Dianat et al., 2014). Our findings now demonstrate that monitoring SOX4 (and SOX9) expression is essential to control the quality of *in vitro*-produced cholangiocyte.

A second strategy is to convert fibroblasts (or another cell type) into cholangiocytes by forced expression of cholangiocyte-specific transcription factors. This method has not yet been achieved to produce *in vitro* cholangiocytes but direct lineage conversion of fibroblast into hepatocytes has been already validated (Huang et al., 2014; Sekiya and Suzuki, 2011; Takayama et al., 2012). Since SOX4 and SOX9 are essential for cholangiocyte differentiation, forced expression of these factors in combination with other biliary-specific transcription factors could be a valuable alternative to produce mature *in vitro* cholangiocytes. A combination of growth factors and forced expression of transcription factors could be considered. Indeed,

Yu et al. have produced bipotential hepatic stem cells using HNF1b and FoxA3, and the cells can then further differentiate into hepatocytic and cholangiocytic lineages following growth factor-mediated programming (Yu et al., 2013). Forcing expression of biliary-specific factors, including SOX4 and SOX9, in bipotential hepatic stem cells would represent yet another alternative to produce cholangiocytes *in vitro*.

References

Antoniou, A., Raynaud, P., Cordi, S., Zong, Y., Tronche, F., Stanger, B.Z., Jacquemin, P., Pierreux, C.E., Clotman, F., Lemaigre, F.P., 2009. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 136, 2325-2333.

Avruch, J., Zhou, D., Fitamant, J., Bardeesy, N., 2011. Mst1/2 signalling to Yap: gatekeeper for liver size and tumour development. British journal of cancer 104, 24-32.

Bhattaram, P., Penzo-Mendez, A., Kato, K., Bandyopadhyay, K., Gadi, A., Taketo, M.M., Lefebvre, V., 2014. SOXC proteins amplify canonical WNT signaling to secure nonchondrocytic fates in skeletogenesis. The Journal of cell biology 207, 657-671.

Bhattaram, P., Penzo-Mendez, A., Sock, E., Colmenares, C., Kaneko, K.J., Vassilev, A., Depamphilis, M.L., Wegner, M., Lefebvre, V., 2010. Organogenesis relies on SoxC transcription factors for the survival of neural and mesenchymal progenitors. Nature communications 1, 9.

Boulter, L., Govaere, O., Bird, T.G., Radulescu, S., Ramachandran, P., Pellicoro, A., Ridgway, R.A., Seo, S.S., Spee, B., Van Rooijen, N., Sansom, O.J., Iredale, J.P., Lowell, S., Roskams, T., Forbes, S.J., 2012. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nature medicine 18, 572-579.

Capaccione, K.M., Hong, X., Morgan, K.M., Liu, W., Bishop, J.M., Liu, L., Markert, E., Deen, M., Minerowicz, C., Bertino, J.R., Allen, T., Pine, S.R., 2014. Sox9 mediates Notch1-induced mesenchymal features in lung adenocarcinoma. Oncotarget 5, 3636-3650.

Carpentier, R., Suner, R.E., van Hul, N., Kopp, J.L., Beaudry, J.B., Cordi, S., Antoniou, A., Raynaud, P., Lepreux, S., Jacquemin, P., Leclercq, I.A., Sander, M., Lemaigre, F.P., 2011. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 141, 1432-1438, 1438 e1431-1434.

Choi, T.Y., Ninov, N., Stainier, D.Y., Shin, D., 2014. Extensive conversion of hepatic biliary epithelial cells to hepatocytes after near total loss of hepatocytes in zebrafish. Gastroenterology 146, 776-788.

Delous, M., Yin, C., Shin, D., Ninov, N., Debrito Carten, J., Pan, L., Ma, T.P., Farber, S.A., Moens, C.B., Stainier, D.Y., 2012. Sox9b is a key regulator of pancreaticobiliary ductal system development. PLoS genetics 8, e1002754.

Dianat, N., Dubois-Pot-Schneider, H., Steichen, C., Desterke, C., Leclerc, P., Raveux, A., Combettes, L., Weber, A., Corlu, A., Dubart-Kupperschmitt, A., 2014. Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells. Hepatology 60, 700-714.

Dill, M.T., Tornillo, L., Fritzius, T., Terracciano, L., Semela, D., Bettler, B., Heim, M.H., Tchorz, J.S., 2013. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology 57, 1607-1619.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S.A., Gayyed, M.F., Anders, R.A., Maitra, A., Pan, D., 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 130, 1120-1133.

Duncan, A.W., Dorrell, C., Grompe, M., 2009. Stem cells and liver regeneration. Gastroenterology 137, 466-481.

Espanol-Suner, R., Carpentier, R., Van Hul, N., Legry, V., Achouri, Y., Cordi, S., Jacquemin, P., Lemaigre, F., Leclercq, I.A., 2012. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 143, 1564-1575 e1567.

Falix, F.A., Weeda, V.B., Labruyere, W.T., Poncy, A., de Waart, D.R., Hakvoort, T.B., Lemaigre, F., Gaemers, I.C., Aronson, D.C., Lamers, W.H., 2014. Hepatic Notch2 deficiency leads to bile duct agenesis perinatally and secondary bile duct formation after weaning. Developmental biology 396, 201-213.

Fiorotto, R., Raizner, A., Morell, C.M., Torsello, B., Scirpo, R., Fabris, L., Spirli, C., Strazzabosco, M., 2013. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. Journal of hepatology 59, 124-130.

Furuyama, K., Kawaguchi, Y., Akiyama, H., Horiguchi, M., Kodama, S., Kuhara, T., Hosokawa, S., Elbahrawy, A., Soeda, T., Koizumi, M., Masui, T., Kawaguchi, M., Takaori, K., Doi, R., Nishi, E., Kakinoki, R., Deng, J.M., Behringer, R.R., Nakamura, T., Uemoto, S., 2011. Continuous cell supply from a Sox9-expressing

progenitor zone in adult liver, exocrine pancreas and intestine. Nature genetics 43, 34-41.

Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimber-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., Siveke, J.T., 2008. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48, 607-616.

Geisler, F., Strazzabosco, M., 2014. Emerging roles of Notch signaling in liver disease. Hepatology.

Hand, N.J., Master, Z.R., Eauclaire, S.F., Weinblatt, D.E., Matthews, R.P., Friedman, J.R., 2009. The microRNA-30 family is required for vertebrate hepatobiliary development. Gastroenterology 136, 1081-1090.

He, J., Lu, H., Zou, Q., Luo, L., 2014. Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. Gastroenterology 146, 789-800 e788.

Huang, P., Zhang, L., Gao, Y., He, Z., Yao, D., Wu, Z., Cen, J., Chen, X., Liu, C., Hu, Y., Lai, D., Hu, Z., Chen, L., Zhang, Y., Cheng, X., Ma, X., Pan, G., Wang, X., Hui, L., 2014. Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. Cell stem cell 14, 370-384.

Jeliazkova, P., Jors, S., Lee, M., Zimber-Strobl, U., Ferrer, J., Schmid, R.M., Siveke, J.T., Geisler, F., 2013. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology 57, 2469-2479.

Kadaja, M., Keyes, B.E., Lin, M., Pasolli, H.A., Genander, M., Polak, L., Stokes, N., Zheng, D., Fuchs, E., 2014. SOX9: a stem cell transcriptional regulator of secreted niche signaling factors. Genes & development 28, 328-341.

Kellendonk, C., Opherk, C., Anlag, K., Schutz, G., Tronche, F., 2000. Hepatocyte-specific expression of Cre recombinase. Genesis 26, 151-153.

Kesavan, G., Sand, F.W., Greiner, T.U., Johansson, J.K., Kobberup, S., Wu, X., Brakebusch, C., Semb, H., 2009. Cdc42-mediated tubulogenesis controls cell specification. Cell 139, 791-801.

Limaye, P.B., Bowen, W.C., Orr, A.V., Luo, J., Tseng, G.C., Michalopoulos, G.K., 2008. Mechanisms of hepatocyte growth factor-mediated and epidermal growth factor-mediated signaling in transdifferentiation of rat hepatocytes to biliary epithelium. Hepatology 47, 1702-1713.

Lincoln, J., Kist, R., Scherer, G., Yutzey, K.E., 2007. Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development. Developmental biology 305, 120-132.

Liu-Chittenden, Y., Huang, B., Shim, J.S., Chen, Q., Lee, S.J., Anders, R.A., Liu, J.O., Pan, D., 2012. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. Genes & development 26, 1300-1305.

Lozier, J., McCright, B., Gridley, T., 2008. Notch signaling regulates bile duct morphogenesis in mice. PloS one 3, e1851.

Malato, Y., Naqvi, S., Schurmann, N., Ng, R., Wang, B., Zape, J., Kay, M.A., Grimm, D., Willenbring, H., 2011. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. The Journal of clinical investigation 121, 4850-4860.

Miner, J.H., Yurchenco, P.D., 2004. Laminin functions in tissue morphogenesis. Annual review of cell and developmental biology 20, 255-284.

Moreno, C.S., 2010. The Sex-determining region Y-box 4 and homeobox C6 transcriptional networks in prostate cancer progression: crosstalk with the Wnt, Notch, and PI3K pathways. The American journal of pathology 176, 518-527.

Paul, M.H., Harvey, R.P., Wegner, M., Sock, E., 2014. Cardiac outflow tract development relies on the complex function of Sox4 and Sox11 in multiple cell types. Cellular and molecular life sciences : CMLS 71, 2931-2945.

Rockich, B.E., Hrycaj, S.M., Shih, H.P., Nagy, M.S., Ferguson, M.A., Kopp, J.L., Sander, M., Wellik, D.M., Spence, J.R., 2013. Sox9 plays multiple roles in the lung epithelium during branching morphogenesis. Proceedings of the National Academy of Sciences of the United States of America 110, E4456-4464.

Rogler, C.E., Levoci, L., Ader, T., Massimi, A., Tchaikovskaya, T., Norel, R., Rogler, L.E., 2009. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. Hepatology 50, 575-584.

Scharer, C.D., McCabe, C.D., Ali-Seyed, M., Berger, M.F., Bulyk, M.L., Moreno, C.S., 2009. Genome-wide promoter analysis of the SOX4 transcriptional network in prostate cancer cells. Cancer research 69, 709-717.

Sekiya, S., Suzuki, A., 2011. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475, 390-393.

Sekiya, S., Suzuki, A., 2014. Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. The American journal of pathology 184, 1468-1478.

Seymour, P.A., Freude, K.K., Tran, M.N., Mayes, E.E., Jensen, J., Kist, R., Scherer, G., Sander, M., 2007. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 104, 1865-1870.

Seymour, P.A., Shih, H.P., Patel, N.A., Freude, K.K., Xie, R., Lim, C.J., Sander, M., 2012. A Sox9/Fgf feed-forward loop maintains pancreatic organ identity. Development 139, 3363-3372.

Shih, H.P., Kopp, J.L., Sandhu, M., Dubois, C.L., Seymour, P.A., Grapin-Botton, A., Sander, M., 2012. A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. Development 139, 2488-2499.

Song, S., Ajani, J.A., Honjo, S., Maru, D.M., Chen, Q., Scott, A.W., Heallen, T.R., Xiao, L., Hofstetter, W.L., Weston, B., Lee, J.H., Wadhwa, R., Sudo, K., Stroehlein, J.R., Martin, J.F., Hung, M.C., Johnson, R.L., 2014. Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. Cancer research 74, 4170-4182.

Strazzabosco, M., Fabris, L., Spirli, C., 2005. Pathophysiology of cholangiopathies. Journal of clinical gastroenterology 39, S90-S102.

Strick-Marchand, H., Weiss, M.C., 2002. Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. Hepatology 36, 794-804.

Takayama, K., Inamura, M., Kawabata, K., Sugawara, M., Kikuchi, K., Higuchi, M., Nagamoto, Y., Watanabe, H., Tashiro, K., Sakurai, F., Hayakawa, T., Furue, M.K., Mizuguchi, H., 2012. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1alpha transduction. Journal of hepatology 57, 628-636.

Tanimizu, N., Kikkawa, Y., Mitaka, T., Miyajima, A., 2012. alpha1- and alpha5-containing laminins regulate the development of bile ducts via beta1 integrin signals. The Journal of biological chemistry 287, 28586-28597.

Tanimizu, N., Miyajima, A., 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. Journal of cell science 117, 3165-3174.

Tanimizu, N., Miyajima, A., Mostov, K.E., 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Molecular biology of the cell 18, 1472-1479.

Tschaharganeh, D.F., Chen, X., Latzko, P., Malz, M., Gaida, M.M., Felix, K., Ladu, S., Singer, S., Pinna, F., Gretz, N., Sticht, C., Tomasi, M.L., Delogu, S., Evert, M., Fan, B., Ribback, S., Jiang, L., Brozzetti, S., Bergmann, F., Dombrowski, F., Schirmacher, P., Calvisi, D.F., Breuhahn, K., 2013. Yes-associated protein up-regulates Jagged-1 and activates the Notch pathway in human hepatocellular carcinoma. Gastroenterology 144, 1530-1542 e1512.

Vervoort, S.J., Lourenco, A.R., van Boxtel, R., Coffer, P.J., 2013. SOX4 mediates TGF-beta-induced expression of mesenchymal markers during mammary cell epithelial to mesenchymal transition. PloS one 8, e53238.

Villanueva, A., Alsinet, C., Yanger, K., Hoshida, Y., Zong, Y., Toffanin, S., Rodriguez-Carunchio, L., Sole, M., Thung, S., Stanger, B.Z., Llovet, J.M., 2012. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 143, 1660-1669 e1667.

Walter, T.J., Vanderpool, C., Cast, A.E., Huppert, S.S., 2014. Intrahepatic bile duct regeneration in mice does not require Hnf6 or Notch signaling through Rbpj. The American journal of pathology 184, 1479-1488.

Wang, H., He, L., Ma, F., Regan, M.M., Balk, S.P., Richardson, A.L., Yuan, X., 2013. SOX9 regulates low density lipoprotein receptor-related protein 6 (LRP6) and T-cell factor 4 (TCF4) expression and Wnt/beta-catenin activation in breast cancer. The Journal of biological chemistry 288, 6478-6487.

Yanai, M., Tatsumi, N., Hasunuma, N., Katsu, K., Endo, F., Yokouchi, Y., 2008. FGF signaling segregates biliary cell-lineage from chick hepatoblasts cooperatively with BMP4 and ECM components in vitro. Developmental dynamics : an official publication of the American Association of Anatomists 237, 1268-1283.

Yanger, K., Stanger, B.Z., 2011. Facultative stem cells in liver and pancreas: fact and fancy. Developmental dynamics : an official publication of the American Association of Anatomists 240, 521-529.

Yanger, K., Zong, Y., Maggs, L.R., Shapira, S.N., Maddipati, R., Aiello, N.M., Thung, S.N., Wells, R.G., Greenbaum, L.E., Stanger, B.Z., 2013. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes & development 27, 719-724.

Yimlamai, D., Christodoulou, C., Galli, G.G., Yanger, K., Pepe-Mooney, B., Gurung, B., Shrestha, K., Cahan, P., Stanger, B.Z., Camargo, F.D., 2014. Hippo pathway activity influences liver cell fate. Cell 157, 1324-1338.

Yu, B., He, Z.Y., You, P., Han, Q.W., Xiang, D., Chen, F., Wang, M.J., Liu, C.C., Lin, X.W., Borjigin, U., Zi, X.Y., Li, J.X., Zhu, H.Y., Li, W.L., Han, C.S., Wangensteen, K.J., Shi, Y., Hui, L.J., Wang, X., Hu, Y.P., 2013. Reprogramming fibroblasts into bipotential hepatic stem cells by defined factors. Cell stem cell 13, 328-340.

Zhang, N., Bai, H., David, K.K., Dong, J., Zheng, Y., Cai, J., Giovannini, M., Liu, P., Anders, R.A., Pan, D., 2010. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Developmental cell 19, 27-38.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., Zheng, P., Ye, K., Chinnaiyan, A., Halder, G., Lai, Z.C., Guan, K.L., 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes & development 21, 2747-2761.

Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigre, F., Stanger, B.Z., 2009. Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727-1739.