Developmental Biology 404 (2015) 136–148

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Transcription factors SOX4 and SOX9 cooperatively control development of bile ducts

Alexis Poncy [1], Aline Antoniou [1,2], Sabine Cordi, Christophe E. Pierreux, Patrick Jacquemin, Frédéric P. Lemaigre *

Université catholique de Louvain, de Duve Institute, Avenue Hippocrate 75/B1-7503, Brussels 1200, Belgium

ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form
22 April 2015
Accepted 15 May 2015
Available online 29 May 2015

Keywords:
SOX transcription factors
Liver development
Biliary tract
Transcriptional network

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.

1. 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; Geisler 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.

* Corresponding author. Fax: +32 27647507.
E-mail addresses: alexis.poncy@uclouvain.be (A. Poncy), aline.antoniou@bordet.be (A. Antoniou), sabine.cordi@uclouvain.be (S. Cordi), christophe.pierreux@uclouvain.be (C.E. Pierreux), patrick.jacquemin@uclouvain.be (P. Jacquemin), frederic.lemaigre@uclouvain.be (F.P. Lemaigre).
[1] These authors contributed equally.
[2] Present address: Institut Jules Bordet, Boulevard Jules Bordet 121-125, 1000 Brussels, Belgium.

http://dx.doi.org/10.1016/j.ydbio.2015.05.012
0012-1606/

Here, we identify SOX4 as a new player in liver development and describe how it regulates bile duct development cooperatively with SOX9.

2. Materials and methods

2.1. 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.

2.2. 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 h 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 (1 g/L) (#F7258, Sigma-Aldrich) and with Direct Red 80 dye (1 g/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.

2.3. 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 (kindly provided by V. Lefebvre), 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 (1 mg/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). The specificity of the in situ hybridization probes was validated previously (Dy et al., 2008; Jacquemin et al., 1996; Lioubinski et al., 2003). Since bile ducts develop according to a hilum-to-periphery axis, several duct maturation levels can be monitored in a single lobe at a single developmental stage. Therefore, during development, the most mature ducts located near the hilum were analyzed.

2.4. 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 RMPI (#31870-025, Gibco, Life technologies) containing collagenase IV (1 mg/mL) (#43E14253, Worthington), dispase (1 mg/mL) (#17105-041, Gibco, Life technologies) and DNase I (0.1 mg/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. Data were normalized to β-actin values.

2.5. 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.

2.6. 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).

2.7. Liver RNA analysis

Wild-type and mutant livers were collected. Total liver RNA was isolated using Trizol (#10296028, Invitrogen, Life technologies) and gene expression was quantified by RT-qPCR with primers listed in Supplementary Table 3. mRNA levels were normalized with β-Actin mRNA.

2.8. Primary cilia quantification

Confocal pictures of acetylated tubulin-stained sections were used to quantify the number of primary cilia at the apical pole of ductal cells in E18.5 fetal livers. The number of ductal cells positive for acetylated tubulin staining was normalized to the number of ductal cells delineating bile ducts. Three animals were analyzed

per genotype and at least 10 hilar bile ducts were studied per animal.

3. Results

3.1. 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 at the onset of biliary development which starts at E11.5–E13.5 (Antoniou et al., 2009). 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 at E13.5 (Fig. 1B and S1), while periportal and parenchymal SOX4 expression was not

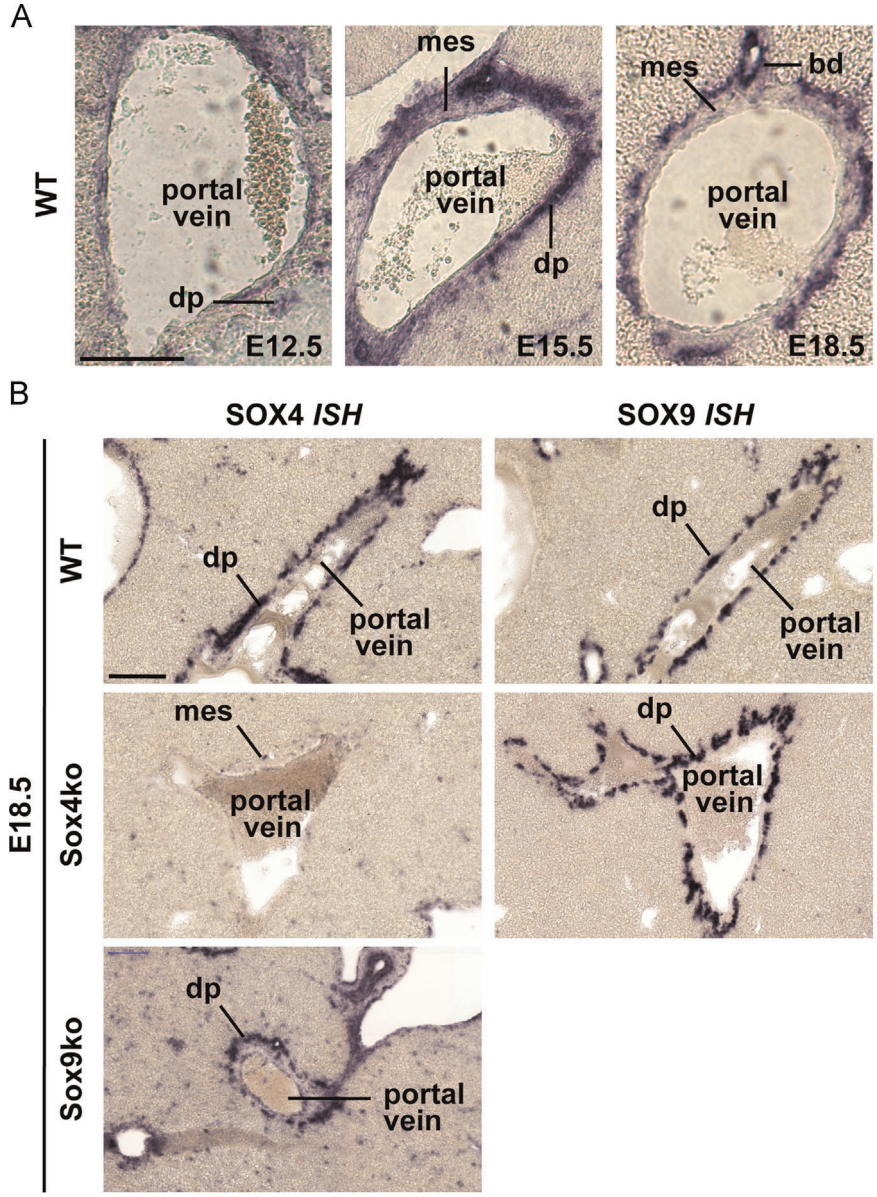

Fig. 1. SOX4 and SOX9 expression in the developing liver. (A) In situ hybridization in WT livers shows SOX4 mRNA expression in portal mesenchyme and biliary cells starting at E12.5. (B) Adjacent tissue sections show overlapping expression of SOX4 and SOX9 in biliary cells. In *Alfp-Cre*;$Sox4^{loxp/loxp}$ (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.

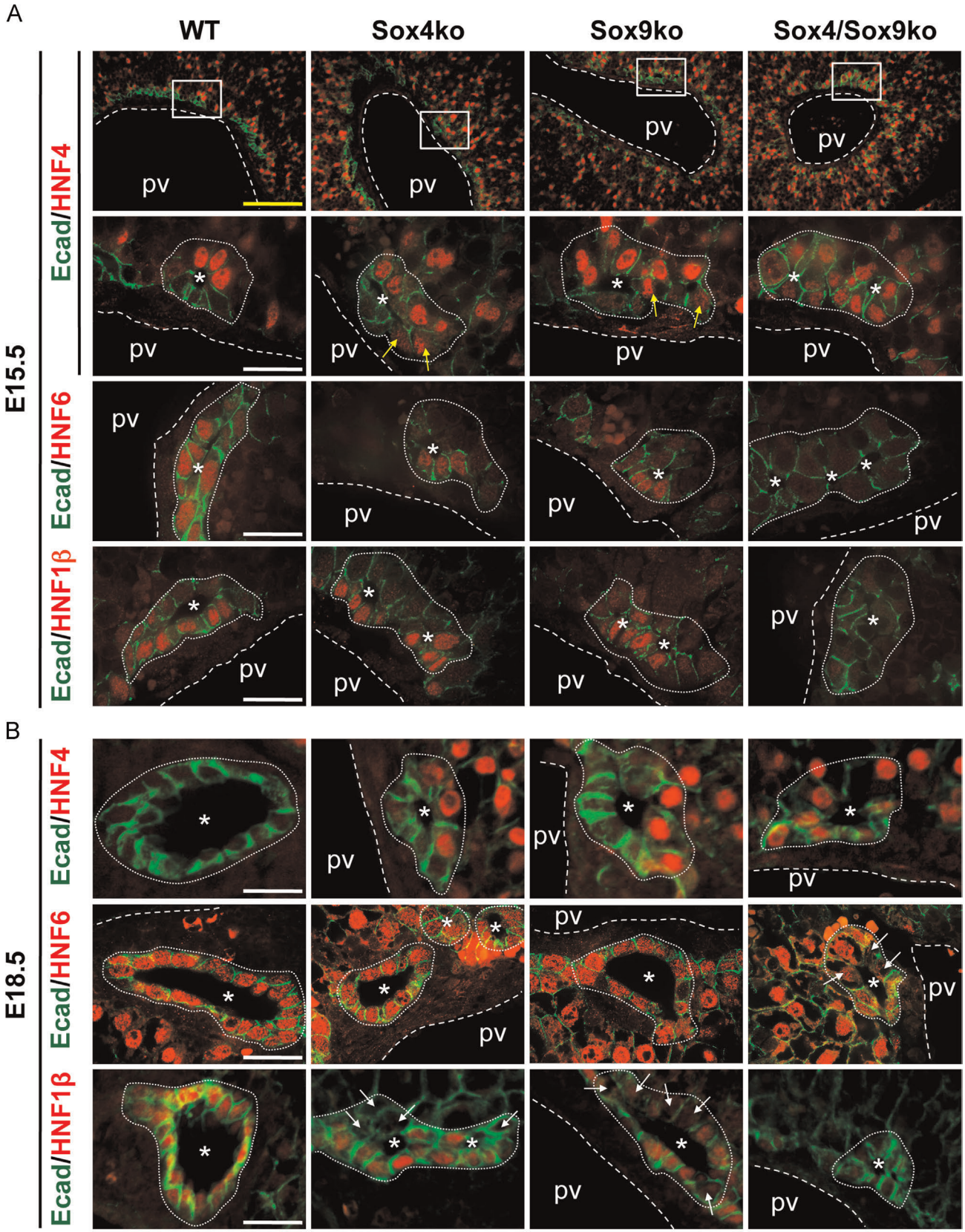

Fig. 2. Cholangiocyte differentiation is perturbed in the absence of SOX4 and SOX9. (A) At E15.5, immunostaining shows residual expression of HNF4 in the portal side of Sox4ko and Sox9ko PDS (yellow arrows) as well as reduced expression of HNF6. In Sox4/Sox9ko, HNF6 and HNF1β expression was not detected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

affected. *Sox9*$^{loxP/loxP}$;*Alfp*-Cre embryos (Sox9ko) showed *Sox9* inactivation starting at E11.5 (Antoniou et al., 2009). Inactivation of either *Sox* gene did not affect expression of the other (Fig. 1B and Fig. S1).

3.2. SOX4 and SOX9 control cholangiocyte differentiation

PDS are detected in wild-type embryos starting at E15.5, and are lined by 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 in single knockouts only induced limited differentiation defects at that stage, namely residual expression of HNF4 in some cells lining the portal side of PDS, lower expression of HNF6 in differentiating cholangiocytes, and normal HNF1β levels on the portal side of the PDS (Fig. 2A). In contrast, the combined inactivation of both factors (*Sox4*$^{loxP/loxP}$;*Sox9*$^{loxP/loxP}$;*Alfp*-Cre (Sox4/Sox9ko)) strongly perturbed biliary differentiation and this was observed in nearly all analysed ducts: biliary markers (HNF6, HNF1β) were barely detectable and expression of the hepatoblast marker HNF4 persisted

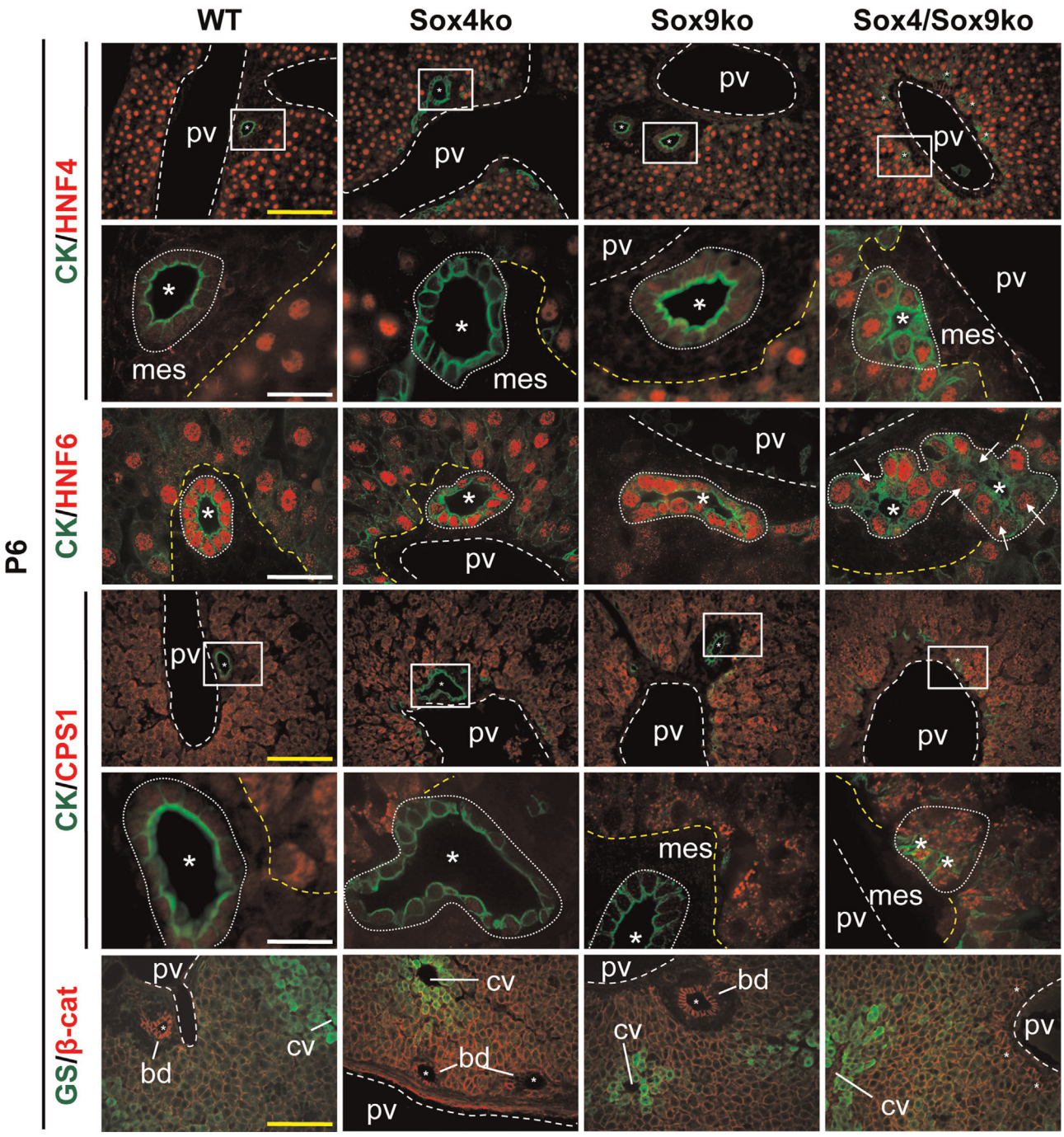

Fig. 3. The lack of SOX4 or SOX9 induces transient defects in bile duct development, while combined absence of both factors is associated with persisting anomalies. Immunostaining at P6 shows normal expression of the differentiation markers HNF4 and HNF6 in Sox4ko and Sox9ko livers. Duct-lining cells in Sox4/Sox9ko liver display hepatocyte characteristics, as shown by expression of HNF4 and carbamoyl phosphate synthase I (CPSI), and low levels of HNF6 (white arrows). Expression of glycogen synthase (GS) was normal and strictly pericentral. β-cat, β-catenin (used as epithelial marker); 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. The yellow dotted line delineates the boundary between hepatocytes and periportal mesenchyme.

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 were FACS-sorted based on Dlk1 expression (Tanimizu et al., 2003) and grown in conditions promoting biliary gene expression, biliary genes were upregulated and hepatocyte markers were decreased after 72 h of culture. In contrast, hepatoblasts purified from Sox4/Sox9ko livers showed lower biliary gene induction and lower repression of hepatocyte

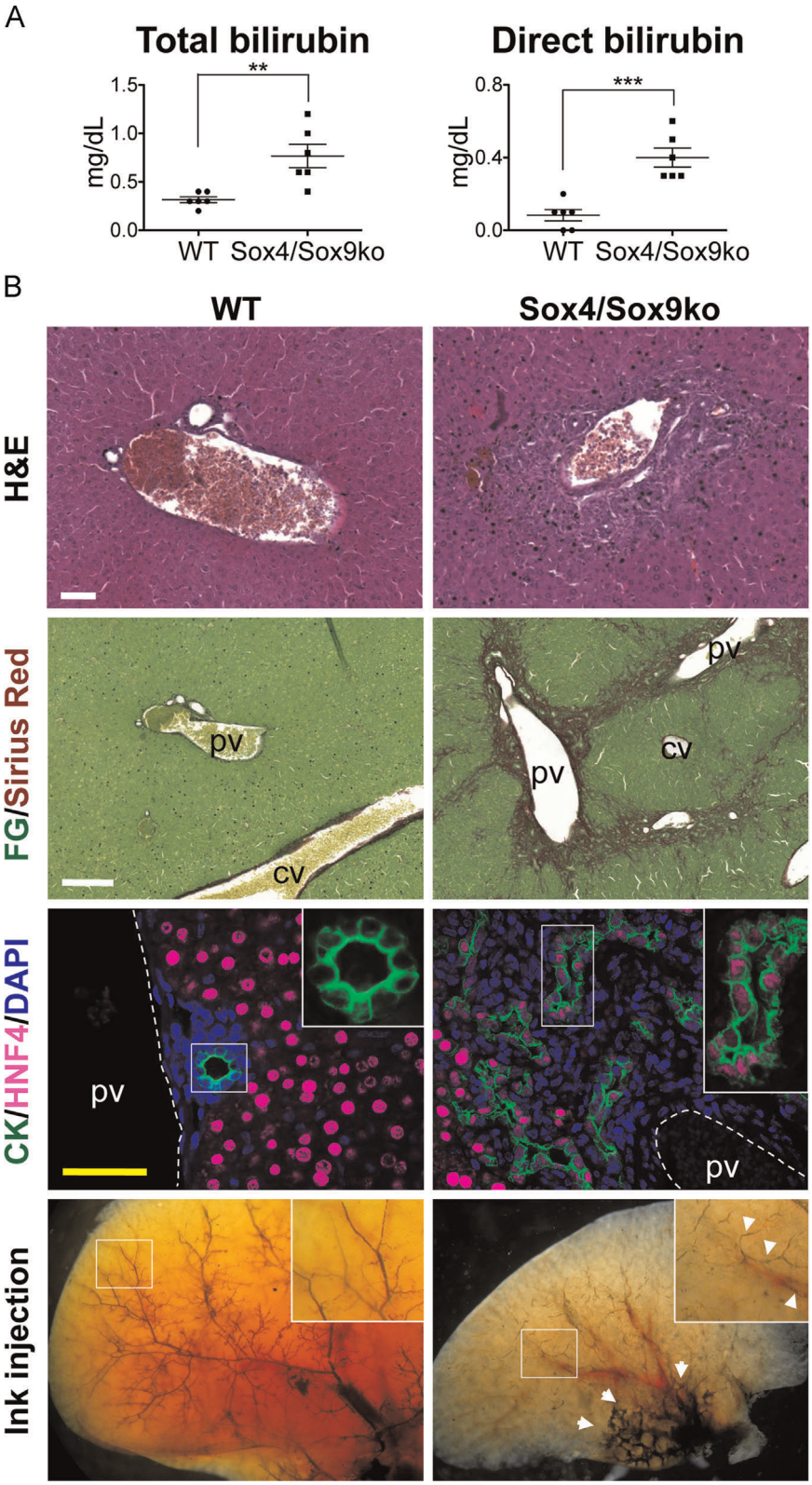

Fig. 4. Six-week-old mice with liver-specific inactivation of SOX4 and SOX9 are cholestatic. (A) Serum levels of bilirubin are increased in Sox4/Sox9ko mice (data are means ± SD; $n = 6$; $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Septal fibrosis and ductular reactions develop as a result from cholestasis 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, FG, Fast green. White size bar, 200 μm; yellow size bar, 50 μm.

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 was still perturbed at E18.5. 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 a significant proportion of biliary cells (mainly on the parenchymal side of ducts) in livers deficient in SOX4 or SOX9, and was barely detectable in double Sox4/Sox9ko knockouts, as compared to wild-type. At E18.5 HNF6 had returned to normal in Sox4ko and Sox9ko biliary cells but was persistently low in a subset of Sox4/Sox9ko duct-lining cells (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 normal 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).

Single and double knockout mice survived well. Six-week-old adult 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.

3.3. 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 single knockouts OPN was initially cytoplasmic (Sox4ko) or nearly undetectable (Sox9ko) at E15.5 but became detectable at the apical pole at the end of gestation (E18.5; Sox4ko and Sox9ko); Mucin-1 was apical, as expected, but very low in a subset of cells at E18.5 (Sox4ko and Sox9ko). RT-qPCR data on whole liver at E18.5 further confirmed these observations, and showed that OPN expression was mainly controlled by SOX9, and Muc1 by SOX4 (Fig. 6A). In contrast, the lack of both SOX factors was associated with absence of apical polarization during gestation (Figs. 5–6A).

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, Mucin-1 was absent at the apical pole of most cells, and a subset of cells did not show Ezrin expression; 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.

3.4. 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. 7). 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. 7). These TβRII-positive cells in the Sox4ko ducts belonged to the cholangiocyte lineage since they were HNF4-negative (Fig. 7). 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. 7). 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.

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 expression, which is specifically detected in biliary cells, depended on SOX4 but not on SOX9: Tead2 was low in Sox4ko and Sox4/Sox9ko livers,

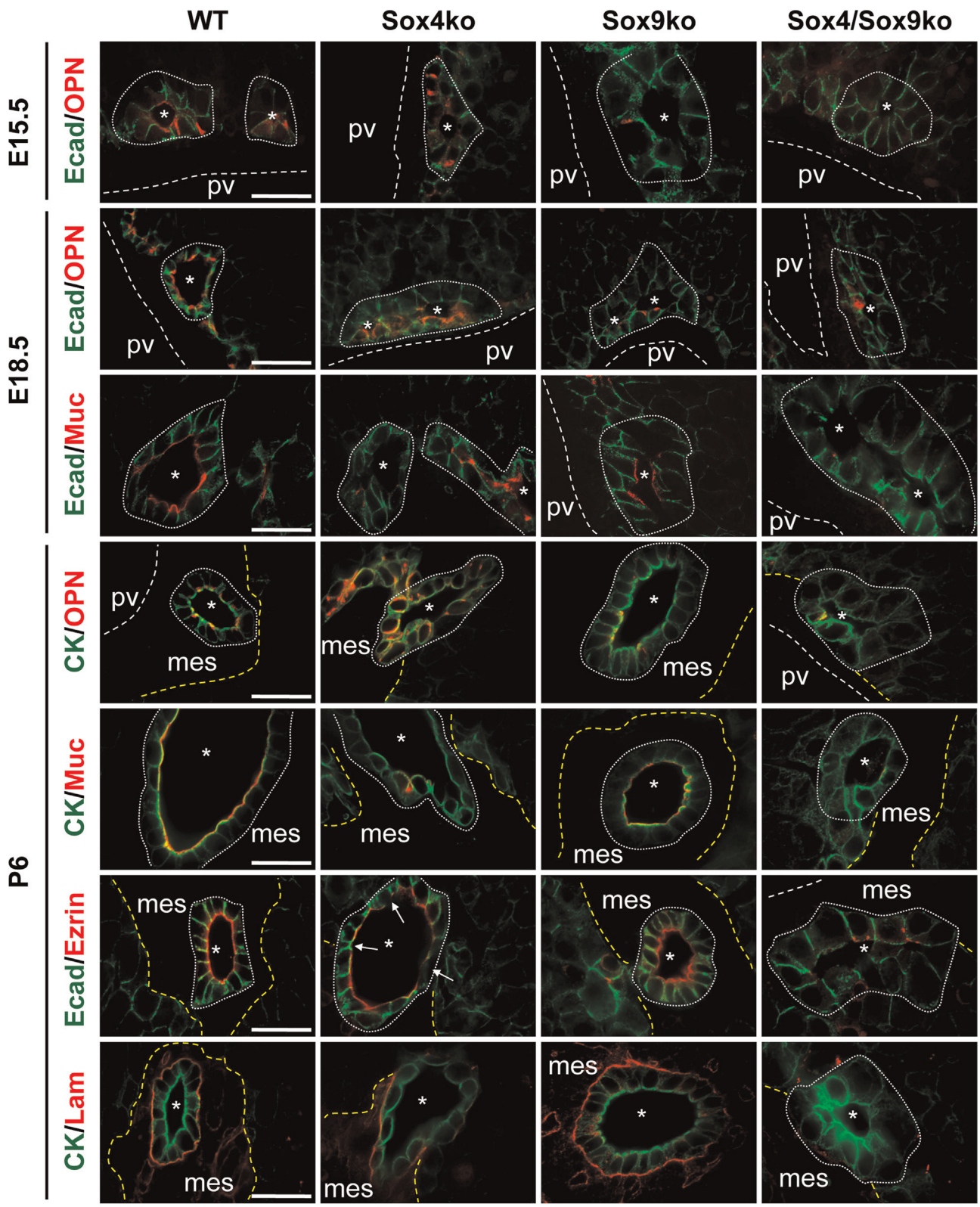

Fig. 5. SOX factors control polarity of cholangiocytes. Immunostaining shows downregulation of apical polarity markers Osteopontin (OPN) and Mucin1 (Muc) in Sox4ko and Sox9ko at E15.5 and E18.5; cell-to-cell variability was observed in the level of OPN and Mucin1 downregulation. Polarity marker expression 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 in a subset of cells (arrows), as well as 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 pan-cytokeratin (CK) staining in Sox4ko and Sox4/Sox9ko. Ecad, E-cadherin; pv, portal vein; mes, mesenchyme; *, lumen of bile ducts. Size bar, 25 μm.

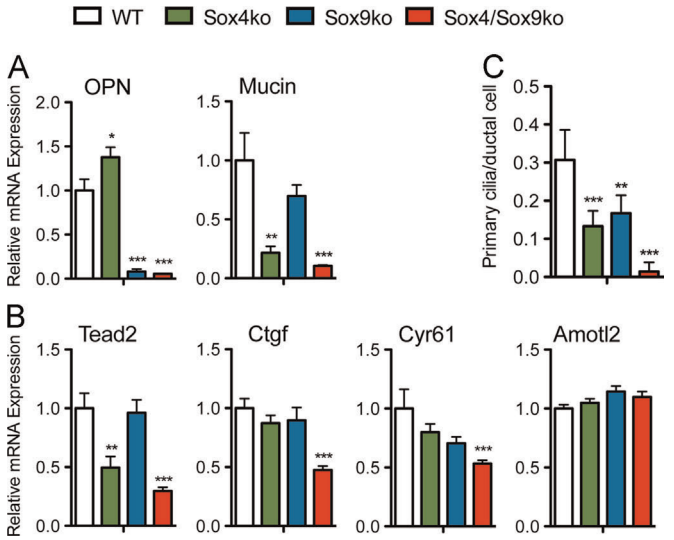

Fig. 6. SOX factors control the expression of polarity and signaling markers, and development of primary cilia. (A) Expression of polarity and (B) signaling markers, measured by RT-qPCR in whole livers at E18.5 (data are means ± SEM; $n=6$ animals per genotype; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). (C) Quantification of primary cilia on duct-lining cells at E18.5 (data are means ± SEM; $n=3$ animals per genotype; $^{**}p<0.01$, $^{***}p<0.001$. From 227 to 420 ductal cells were counted per genotype).

but not in Sox9ko livers (Figs. 6B and 7). This was also seen in cultured hepatoblasts deficient in SOX4 and SOX9 (Fig. S2). When we analyzed the expression of Hippo/Yap signaling markers Ctgf, Cyr61 and Amotl2 (Yimlamai et al., 2014), we found that inactivation of either of the two SOX factors had no significant impact, but that two of the markers were downregulated in the combined absence of SOX4 and SOX9 (Fig. 6B). We concluded that the SOX factors may participate to the regulation of Hippo signaling, but that this does not seem to be critical for SOX factor function.

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., 2014). Cilia were detected by staining for acetylated tubulin in livers deficient in SOX4 or SOX9 at prenatal and postnatal stages (Fig. 7 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 (Figs. 6C, 7 and 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). Laminin α1 was not affected by the absence of SOX factors. In contrast, in single and double knockouts laminin α5 was lacking along the parenchymal side of bile ducts (Fig. 7). 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.

4. 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/Sox9ko 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 (Fig. 8).

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. We note that Hes1 expression, like HNF1β expression, is predominantly reduced at the parenchymal side of ducts. This suggests that aberrant Hes1 and HNF1β expression reflects deficient maturation of the parenchymal side of the ducts. Since Notch signaling functions by cell–cell contact, aberrant Notch signaling may cause cell-to-cell variability in gene expression. This may contribute to the cell-to-cell variability in expression of proteins such as Ezrin, HNF1β or HNF6.

Furthermore, 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

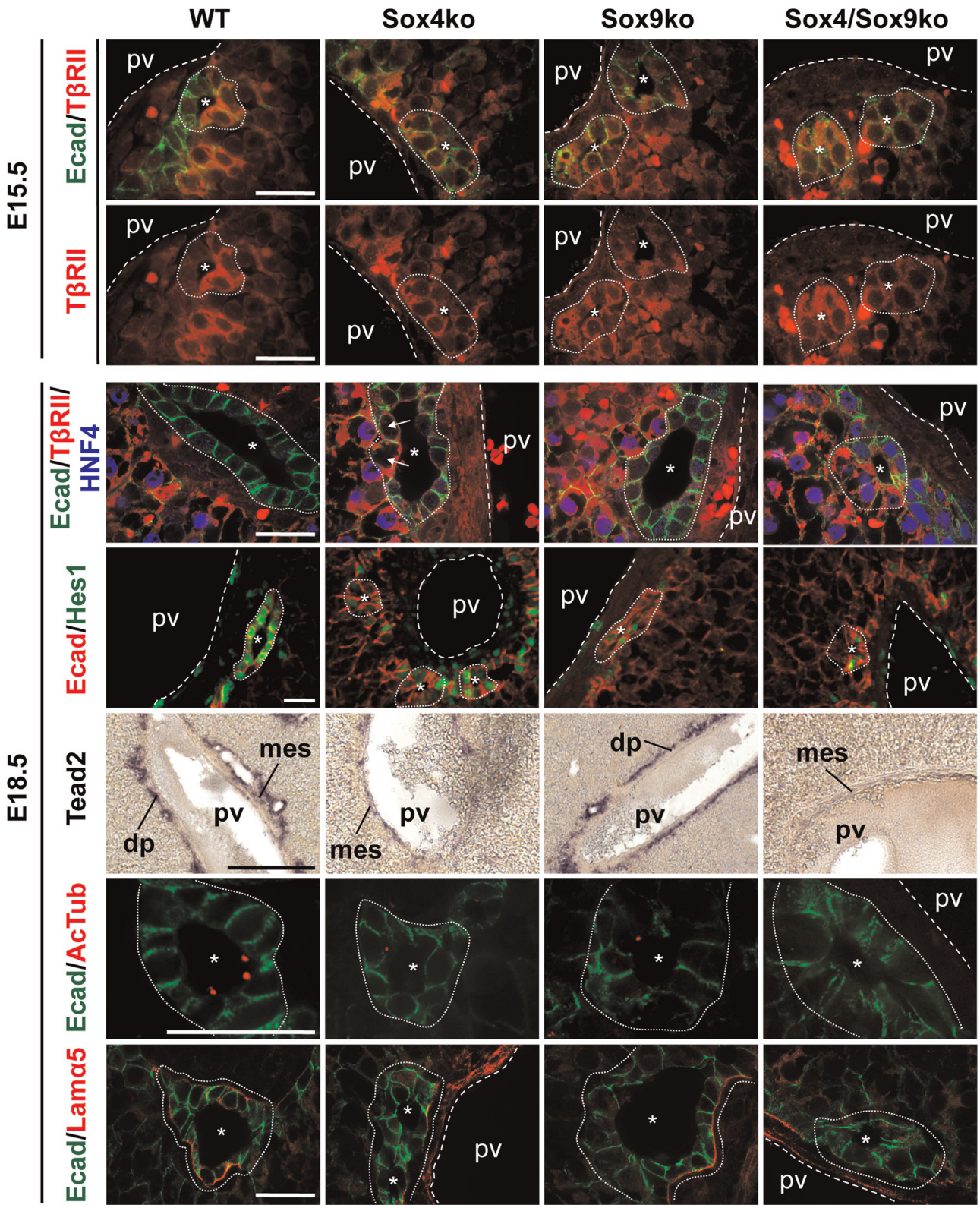

Fig. 7. SOX factors control signaling pathways involved in biliary development. At E15.5, TβRII is repressed on the portal side of PDS in wild-type livers but persists in all PDS cells of knockout mice. At E18.5, TβRII is downregulated in wild-type ducts but still detectable in a subset of cells lining Sox4ko (white arrows) 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 absent from to the parenchymal side of bile ducts in Sox4ko, Sox9ko and Sox4/Sox9ko livers. 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.

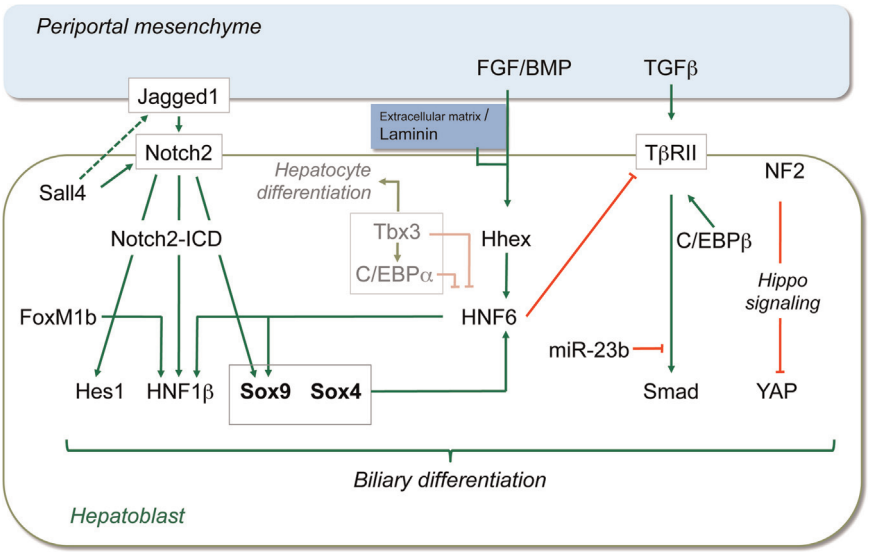

Fig. 8. Schematic overview of gene interactions operating during differentiation of hepatoblasts to cholangiocytes. The scheme integrates data from references Clotman et al. (2005, 2002), Geisler et al. (2008), Hunter et al. (2007), Krupczak-Hollis et al. (2004), Ludtke et al. (2009), Oikawa et al. (2009), Rogler et al. (2009), Takayama et al. (2014), Tanimizu and Miyajima (2004), Tanimizu et al. (2007), Tchorz et al. (2009), Zhang et al. (2010), Zong et al. (2009).

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 on Notch signaling mediators cannot be excluded in biliary development. Along the same lines, primary cilia are absent at the apical pole of cholangiocytes in liver in combined absence of SOX4 and SOX9. Since primary cilia can modulate Notch and TGF-β signaling (Clement et al., 2013; Leitch et al., 2014), 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, but does not require SOX9. Importantly, downregulation of Tead2 in Sox4ko liver is neither associated with changes in the nuclear localization of YAP, nor with downregulation of YAP target genes. This raises the possibility that other Tead proteins compensate for the reduced levels of Tead2. Livers doubly deficient in SOX4 and SOX9 show reduced levels of a subset of Hippo signaling markers, without anomaly of nuclear YAP location. This raises the possibility that SOX factors regulate Hippo signaling during bile duct development, but our data do not support that a SOX-Hippo cascade is a master regulator of biliary development.

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 adult animals, the absence of SOX4 and SOX9 is associated with deficient development of peripheral bile ducts, but persistence of dilated hilar ducts. This observation suggests that the cholangiocytes lining hilar and peripheral ducts belong to distinct subtypes. Whether this reflects a distinct embryonic origin cannot be determined. Intrahepatic cholangiocytes derive from hepatoblasts, while extrahepatic cholangiocytes derive from a diverticulum of the endoderm. Where the intra- and extrahepatic ducts fuse is not known, and we cannot eliminate the possibility that the persisting hilar ducts in Sox4/Sox9ko liver derive from extrahepatic ducts. In any case, these hilar ducts were deficient in SOX factors (not shown).

Ductular reactions were detected in adult Sox4/Sox9ko liver. This is most likely induced by chronic cholestasis, but the origin of the ductular reactive cells has not been determined. Such cells may derive from the biliary tract or from hepatocytes. We favor the hypothesis that they derive from hepatocytes since most ductular reactive cells express the hepatocyte marker HNF4.

To conclude, we found that SOX factors cooperate to regulate bile duct development and propose that they are integrated in the biliary gene network (Fig. 8) 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.

Acknowledgments

This work was supported by the Interuniversity Attraction Pole Programme (Belgian Science Policy, PVII-47), the D.G. Higher Education and Scientific Research of the French Community of Belgium (ARC 10/15-029), the Commission of the European Communities (BOLD project (grant 238821)), the Alphonse and Jean Forton Fund, the Université Catholique de Louvain and the F.R.S.-FNRS (Belgium: Grants 3.4536.1 and J.0058.15). CEP and PJ are research associates of the F.R.S.-FNRS. AP held a fellowship from Télévie. The authors thank M. Sander, F. Tronche for mice, V. Lefbvre for mice, material and advice, T. Sasaki for anti-laminin antibodies, Bianca Pastorelli for technical assistance, J.B. Beaudry and the members of the Lemaigre lab for help and discussions.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at 10.1016/j.ydbio.2015.05.012

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. Nat. Commun. 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 Dev. 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.

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 Genet. 8, e1002754.

Dy, P., Penzo-Mendez, A., Wang, H., Pedraza, C.E., Macklin, W.B., Lefebvre, V., 2008. The three SoxC proteins—Sox4, Sox11 and Sox12—exhibit overlapping expression patterns and molecular properties. Nucleic Acids Res. 36, 3101–3117.

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. J. Cell Biol. 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. J. Biol. Chem. 283, 14063–14071.

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. Dev. Biol. 308, 355–367.

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. J. Biol. Chem. 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.

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. Dev. Biol. 276, 74–88.

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. Nat. Immunol. 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 facto. Int. J. Biochem. Cell Biol. 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. J. Cell Sci. 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. Dev. Biol. 305, 120–132.

Lioubinski, O., Muller, M., Wegner, M., Sander, M., 2003. Expression of Sox transcription factors in the developing mouse pancreas. Dev. Dyn. 227, 402–408.

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.

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. Dev. Biol. 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. Am. J. Pathol. 176, 518–527.

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.

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. Int. J. Biochem. Cell Biol. 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. Proc. Natl. Acad. Sci. USA 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 Res. 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. Proc. Natl. Acad. Sci. USA 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. Dev. 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 Res. 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. J. Biol. Chem. 287, 28586–28597.

Tanimizu, N., Miyajima, A., 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J. Cell Sci. 117, 3165–3174.

Tanimizu, N., Miyajima, A., Mostov, K.E., 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol. Biol. Cell 18, 1472–1479.

Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T., Miyajima, A., 2003. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell Sci. 116, 1775–1786.

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. J. Vis. Exp., e4272.
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. Dev. 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.