DLK1 and the Notch pathway in the liver

Falix, F.A.

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NOTCH2 IS REQUIRED FOR CHOLANGIOCYTE DIFFERENTIATION

CHAPTER 6
Abstract

The Notch pathway 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<sup>fl/fl</sup>/Alfp-Cre<sup>tg/-</sup> ("Notch2-cKO") and Notch2<sup>fl/fl</sup>/Alfp-Cre<sup>-/-</sup> ("control") mice. Neonatal Notch2-cKO livers were completely devoid of cytokeratin19 (CK19)-positive ductal structures, demonstrating absence of embryonic cholangiocyte differentiation. Despite extensive cholestatic necrosis, mortality was only ~15%. Surprisingly, isolated CK19- and annexinIV-positive cells appeared near portal tracts after weaning and a few small bile ducts were formed at 6 weeks. Despite extensive liver fibrosis, jaundice had disappeared in ~30% of Notch2-cKO mice by 6 months. Bile ducts formed postnatally resembled the atypical ductular reaction histologically. Notch2 and Hnf6 mRNA levels were permanently decreased in Notch2-cKO livers, implying that Hnf6 depends on Notch2 signaling. Foxa1, Foxa2, Hhex, Hnf1β, Cebpα and Sox9 mRNA levels were all significantly lower than controls perinatally, 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. Expression of the Fxr target Shp was elevated at 6 weeks, but no longer at 6 months, suggesting that bile acids are necessary to initiate, but not to expand secondary bile duct development. Conclusion: Notch2 deficiency causes bile duct agenesis, yet allows for slow, secondary bile duct formation after weaning that resembles the atypical ductular reaction.

Farah A. Falix1,2, Viola B. Weeda1,2, Wilhelmina T. Labruyere1, Dirk R. de Waart1, Theodorus B.M. Hakvoort1, Ingrid C. Gaemers1, Daniël C. Aronson2, Wouter H. Lamers1

1 Tytgat Institute for Liver and Intestinal Research, and
2 Pediatric Surgical Center of Amsterdam, Emma Children’s Hospital AMC, Academic Medical Center, Amsterdam, The Netherlands

Keywords
Notch2, ductal plate, cholangiocyte

List of abbreviations
cKO, conditional knockout; ED, Embryonic day; ND, neonatal day; D, postnatal day; CK19, cytokeratin 19
Introduction

In mice, the hepatic diverticulum is formed on embryonic day (ED) 9 and gives rise to the liver and all intrahepatic bile ducts (1, 2). Half a day later, hepatoblasts separate from the diverticulum and invade the adjacent septum-transversum mesenchyme (1). These hepatoblasts are bipotential and can either differentiate into hepatocytes or cholangiocytes. The developing intrahepatic biliary tree is continuous with the extrahepatic bile ducts (3, 4).

The morphogenesis of the intrahepatic bile ducts is similar in humans and rodents, and proceeds stepwise (5). In ED14.5 mice, the hepatoblasts bordering the large portal tracts begin to express cholangiocyte-specific marker proteins (cytokeratin19 (CK19) and EpCAM) and transform the next day into the single-cell layered "ductal plate" (5, 6). Around ED15.5, the ductal plate becomes bilayered and begins to remodel by forming focal dilations between the two cell layers to form the lumina of "primitive ductular structures". The parts of the ductal plate that do not form patent ducts regress. In the final stage, which starts around birth, the ducts are incorporated into the portal mesenchyme (5). The process of intrahepatic bile duct morphogenesis proceeds along a gradient from the hilum to the periphery of the liver, so that the smallest portal-vein branches in the liver periphery are still surrounded by ductal plates at birth (2).

The Notch signaling pathway is highly conserved throughout evolution and plays an important role in cell-fate determination by way of cell-cell contacts. Mammals express four Notch receptors (Notch1-4) with five canonical ligands (Delta-like ligand 1 (Dll1), Dll3, Dll4, Jagged1 and Jagged2). When a ligand binds a Notch receptor, proteolytic cleavage releases the Notch intracellular domain from the membrane, so that it can enter the nucleus and form a transcriptionally active complex with DNA-binding partner Rbp-j (Recombination signal-binding protein for immunoglobulin kappa J region) and a tissue-specific transcription factor (7). The best established role for the Notch pathway in liver development is the involvement of NOTCH2 and JAGGED1 in Alagille syndrome, a rare hereditary disorder with multiple developmental abnormalities, including bile duct paucity (8, 9). Liver-specific Notch2 deficiency or haplo-insufficiency of Jagged1 and Notch2 in mice is sufficient to induce the bile duct abnormalities seen in Alagille syndrome (8, 10, 11). Two studies (10, 11) used transgenic Albumin-Cre mice to bring about liver-specific deletion of Notch2. Since this transgene becomes expressed late embryonically (12), probably after the time point of hepatobiliary differentiation, it was concluded that Notch2 is required for proper intrahepatic bile duct morphogenesis, but its role in cholangiocyte fate decisions remained unclear (10, 11). Liver-specific deletion with Foxa3-Cre or Alfp-Cre transgenes should lead to deletion before the onset of hepatobiliary differentiation, with Cre recombinase activity becoming active around ED8.5 and ED10.5, respectively (13, 14). Early liver-specific deletion of the Notch DNA binding partner Rbp-j with Foxa3-Cre caused a reduction in the number of ductal-plate cells at ED16.5 and postnatal day 1 (D1), and a significant decrease in the number of bile ducts on ND1, whereas later ablation with Alfp-Cre left ductal-plate formation intact, but caused a significant reduction in the number of bile ducts.
postnatally (6). These findings show that the Notch2/Rbp-J complex controls multiple steps in biliary development, including the initial differentiation of cholangiocytes, but they are not conclusive with respect to the role of Notch2 in determining cholangiocyte differentiation.

In the present study, we investigated the consequences of Notch2 ablation before the onset of hepato-biliary differentiation by crossing Notch2<sup>fl/fl</sup> mice with Alfp-Cre mice. In Alfp-Cre mice, the addition of the far-upstream α-Fetoprotein enhancer elements to the Albumin promoter mediates Cre recombinase expression and activity around ED10.5 (13), which is well before the initiation of intrahepatic bile duct formation. We were able to show that Notch2 is indispensable for cholangiocyte differentiation and subsequent ductal plate formation. Unexpectedly, we found that these mice developed a Notch2-and Hnf6-independent program of cholangiocyte differentiation after weaning and that this program involved most of the other transcription factors associated with embryonic bile duct development. The small functional network that had developed was sufficient to resolve the icteric state in ~30% of cases.
Materials and Methods

Animals
All mice (mixed FVB/C57Bl6 background) were maintained on a 12h light/12h dark cycle with free access to water and food. For early hepatocyte-specific deletion of Notch2, Notch2fl/fl mice (15) were crossed with Alfp-Cre mice (13). Toe DNA was used to genotype Notch2fl/fl/Alfp-Cre−/− mice (further designated Notch2-cKOs) and their Notch2fl/fl/Alfp-Cre−/− littermates (controls). Primer sequences are given in Table S1 in supplementary material. The studies were carried out in accordance with Dutch guidelines for the Care and Use of Laboratory Animals and approved by the AMC supervisory committee.

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<th>primers used for qRT-PCR analysis 5′-3′</th>
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<td>TENGT (10mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.025% (w/v) gelatin, 0.05% (v/v) Tween-20) and incubated overnight with polyclonal rabbit antibodies against cytokeratin 19 (CK19; home-made), annexinIV (17), Ki67 (ab15580 Abcam, Cambridge UK), and Carbamoylphosphate Synthetase (CPS; (18)) diluted 1:1,000, 1:5,000, 1:400, and 1:1,000, respectively, in TENGT. Monoclonal Glutamine Synthetase (GS; BD Transduction Laboratories Breda, The Netherlands) was diluted 1:1,000 in TENG. After washing 3 times in Phosphatase-Buffered Saline (PBS), sections were incubated with alkaline phosphatase-labeled goat-anti-rabbit antibody</td>
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Tissue collection
For embryonic liver isolation, noon of the day of the detection of a vaginal plug was designated as embryonic day (ED) 0.5. To confirm the gestational age, the crown-rump length of the embryo was measured and compared with the Table of Rugh (16). Livers from Notch2-cKOs and their littermate controls were collected on ED16.5, neonatal day 1 (D1), postnatal day 21 (D21), D42, D180 and D365+ for histology, immunohistochemistry and RNA isolation.

Histology and Immunohistochemistry
Livers were fixed overnight in 4% buffered formaldehyde, embedded in paraffin and sectioned at 4 or 7 µm thickness. For histological examination, 4µm sections were stained with Hematoxylin & Eosin (H&E), Sirius red or PAS. For immunohistochemistry, 7 µm sections (immunohistochemistry) were deparaffinized, hydrated in graded alcohols, heated for 10 min at 120º C, 1 kPa in 10 mM sodium citrate (pH 6.0) to retrieve antigens, blocked in TENGT (10mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.025% (w/v) gelatin, 0.05% (v/v) Tween-20) and incubated overnight with polyclonal rabbit antibodies against cytokeratin 19 (CK19; home-made), annexinIV (17), Ki67 (ab15580 Abcam, Cambridge UK), and Carbamoylphosphate Synthetase (CPS; (18)) diluted 1:1,000, 1:5,000, 1:400, and 1:1,000, respectively, in TENG. Monoclonal Glutamine Synthetase (GS; BD Transduction Laboratories Breda, The Netherlands) was diluted 1:1,000 in TENG. After washing 3 times in Phosphatase-Buffered Saline (PBS), sections were incubated with alkaline phosphatase-labeled goat-anti-rabbit antibody
Materials and Methods

(A418 Sigma, Zwijndrecht, The Netherlands), diluted 1:200 in TNEG, for 1.5 hour or goat-anti-mouse antibody (Sigma), diluted 1:100 in TNEG. The sections were then washed 3 times in PBS, followed by visualization of bound alkaline phosphatase with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP 1:50; Roche, Woerden, The Netherlands). After quick dehydration in graded alcohols, the sections were mounted with Entellan (Merck, Darmstadt, Germany) and photographed with a Leica DMRA2 microscope equipped with a DC300 camera.

Gallbladder cannulation and resin casting of intrahepatic bile ducts
Six-month old mice were anaesthetized by intraperitoneal injection of 0.1 mL/5 g body weight FFD (hypnorm (fentanyl/fluanisone) and diazepam) and placed on a heat pad to maintain body temperature. After opening the abdomen and ligating the common bile duct, the gallbladder was cannulated with 4 cm "Fine-Bore Polyethylene Tubing” (0.4 (ID)*0.8 (OD) mm; Smiths Medical International, Hytte, Kent, UK). The cannula was fixed with a ligature and Histoacryl tissue glue (n-butyl-2 cyanoacrylate; B. Braun Melsungen AG, Germany). Bile was collected for 45 min. The bile duct tree was visualized by resin casting as described (19): 75-100 µL liquid Mercox II Resin (20 mg resin mixed with 0.5 mL benzoyl peroxide (40%); Ladd Research, Williston, VT, USA) was pumped retrogradely at 25 µL/min into the bile duct tree. After polymerization, the entire liver was removed and placed in warm tap water for 10-30 minutes for curing, followed by overnight maceration of the liver tissue at room temperature in 15% KOH. Casts were rinsed with water.

Analysis of bilirubin and bile salts in bile and plasma.
Concentrations of bilirubin mono- (BMG) and diglucuronide (BDG), unconjugated bilirubin (UCB), and bile salts in bile and plasma were determined by reverse-phase HPLC (20). In brief, 100 µL diluted bile or deproteinized plasma was applied to a Hypersil C18, 3 µm, 15 cm HPLC column (Thermo Scientific, Breda, The Netherlands). The starting eluent consisted of 6.8 mM ammoniumformate (pH 3.9), followed by several steps of linear gradients of acetonitrile (Biosolve, Valkenswaard, The Netherlands). Detection was performed using a Nano Quantity Analyte Detector (NQAD) QT-500 (Quant technologies, Blaine, USA). The respective bile-salt species were identified using calibration curves.

Statistics
Data are shown as mean ± SEM of 4-9 animals per group. Statistical significance of differences was determined by the Kruskal-Wallis one-way ANOVA nonparametric test. Significance was set at P values ≤0.05.
Quantitative PCR

Total liver RNA was isolated using the Trizol reagent (Invitrogen, Breda, The Netherlands). Two µg purified RNA was reverse transcribed with SuperscriptIII Reverse Transcriptase (Invitrogen, Breda, The Netherlands). Quantitation was carried out in the LC480 apparatus with SYBR green (Roche). Primers were intron spanning, designed with Primer3 software at the supplied default settings (http://frodo.wi.mit.edu). Primer sequences are given in Table S2 in the supplemental material. mRNA levels were quantified using an established algorithm (21). This algorithm estimates the baseline by reconstructing the log-linear phase downward from the early plateau phase of the PCR reaction and determines PCR efficiency per sample by fitting a regression line to the data points in the log-linear phase. The mean of these PCR efficiencies per amplicon is used in the calculation of the starting mRNA concentration of the samples. mRNA levels were normalized with 18S rRNA.

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Table S2 Primers used for qPCR

All qPCR primers were designed to span an intron.
Results

Early liver-specific deletion of Notch2 leads to agenesis of intrahepatic bile ducts

Excision of the Notch2 gene in crosses of Alfp-Cre and Notch2\textsuperscript{fl/fl} mice was examined at ND1 and only observed in genomic DNA of Notch2-cKO livers (Figure 1A). Notch2-cKO mice were born at the expected Mendelian frequency, but were ~30% smaller than their control littermates. The growth retardation persisted throughout life (Figure 1B, C).

![Figure 1](image1)

Figure 1 Liver-specific excision of Notch2 and phenotype of Notch2-cKO mice
Panel A: PCR analysis of Notch2\textsuperscript{fl/fl} alleles in genomic DNA at D1. Lanes 1, 2: 1,500 bp band of intact Notch2 allele in control liver and spleen, respectively; lane 3: 500 bp band of deleted Notch2 allele in a Notch2-cKO liver; lane 4: intact Notch2 allele in the corresponding spleen. Asterisks: nonspecific band in control mice. Panel B: Notch2-KO mouse (left) and a control littermate (right) on D21. Panel C: early Notch2 deletion causes permanent ~35% reduction in body weight of Notch2-cKOs (black bars) compared to controls (white bars) (*: p<0.05).

![Figure 2](image2)

Figure 2 CK19 expression in ED16.5 livers of Notch2-cKO and control mice
Panel A: control liver with ductal plate as single-cell, CK19+ layer around large portal veins (p) near liver hilum (arrows: CK19+ extrahepatic bile ducts; arrowheads: CK19+ liver capsule). Panel B: large portal vein in different ED16.5 control liver, surrounded by both CK19+ single layered ductal plate (magnification: orange inset) and developing luminal structures (magnification: black inset). Panel C: ED16.5 Notch2-cKO liver with total absence of CK19+ structures around large portal vein near hilum. Note presence of CK19+ epithelium of esophagus (e) and liver capsule (arrowhead). c: central vein.
Results

To assess intrahepatic bile duct formation, livers of Notch2-cKO and control mice were stained for the biliary markers CK19 and AnnexinIV, which showed comparable staining patterns. Control livers showed extensive ductal plate formation on ED16.5, with already a few tubular structures around large portal veins near the liver hilum (Figure 2A, B) and on ND1 both mature bile ducts and ductal plate remnants were present around the large portal veins (supplemental Figure S1a-c). In contrast, in Notch2-cKO livers a complete absence of ductal plate formation was seen in ED16.5 (Figure 2C), with absence of bile ducts at ND1 (supplemental Figure S1d-h). Liver-specific Notch2 ablation did not affect extrahepatic bile duct development (Figure S1h in supplemental material). Despite the complete lack of bile ducts, the majority of Notch2-cKOs survived, with an overall mortality of only ~15% (12 deaths among 95 Notch2-cKO mice) during the first 6 postnatal weeks.

Figure S1 CK19 expression in D1 liver of Notch2-cKO and control mice
Panels a, d, and g: sections stained for the presence of glutamine synthetase (GS) of D1 control (a), and Notch2-cKO (d,g) livers. Note selective staining of GS in pericentral hepatocytes; portal veins are marked by asterisks. Panels b, c: serial sections of panel a stained for annexinIV and CK19, respectively. Portal veins (asterisks) are associated with one bile duct and ductal plate remnants. Note similarity of annexinIV and CK19 staining, except for higher background in annexinIV-stained sections. Panels e, f (serial sections of panel d): total absence of annexinIV and CK19 staining, respectively, around portal veins in Notch2-cKO livers. Panel h (serial section of panel g): D1 Notch2-cKO liver stained for the presence of CK19, with only the extrahepatic bile duct expressing CK19. Scale bar in panel a is applicable to panels b-h.
Results

Notch2 deletion causes cholestatic necrosis in the liver, but permits secondary bile duct formation after weaning

In D21 control livers, only mature bile ducts were found (Figure 3A). All D21 Notch2-cKO mice that were sacrificed (n=9) were severely icteric. While the parenchymal tissue surrounding some large portal veins in D21 Notch2-cKO livers was still completely devoid of CK19-positive cells (Figure 3B), other large portal veins revealed the presence of scattered CK19-positive cells (Figure 3C) that resembled those normally seen in early stages of ductal-plate formation. Inspection of the surface and sections of livers of D21 Notch2-cKO mice also revealed multiple pale patches (Figure 3B, marked by n) that were due to necrosis (see next section). Irrespective of their serious cholestatic condition, some of D42 Notch2-cKO livers showed, when compared with D21 Notch2-cKOs, further development of CK19-positive ductal plate-like structures into tubular structures around large portal veins (Figure 3D-E), indicative of ongoing bile duct formation.

Figure 3 CK19 expression in D21 and D42 livers of Notch2-cKO and control mice
Panels A, D: control livers of D21 and D42 mice, respectively, with CK19+ bile ducts around large portal veins (p). Panel B: D21 Notch2-cKO livers may (B) or may not (C) have developed short, single cell-layered, CK19+ strands around portal veins (arrows in C and magnification in inset). Note pale, necrotic area (n) in B. Panels E, F: D42 Notch2-cKO livers contain both single CK19+ cell-layered strands (E and magnification in inset) and luminal structures (F and magnification in inset). Panel G: area around large portal vein of Notch2-cKO liver with irregular CK19+ cell strands resembling ductal-plate formation. p: portal vein; c: central vein. Scale bar in panel A is applicable to panels B-F.
Routine H&E-stained histology revealed varying stages of necrosis in D42 Notch2-cKOs. Established lesions were pale, with the cellular membrane and nucleus still vaguely visible, whereas fresh, expanding lesions stained darker and were surrounded by 2-4 rows of densely staining, dying hepatocytes. The fresh lesions were surrounded by inflammatory infiltrates (Figure 4A). These features are typical for cholestatic hepatocyte injury (22). In agreement with this conclusion, serum alkaline phosphatase (AF), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) levels were 5-10-fold increased in D21 and D42 Notch2-cKOs (data not shown).

The size of the liver of newly weaned and adolescent Notch2-cKO mice was ~2-fold bigger than that of control mice, with the biggest increase seen at 6 weeks (Figure 4B). To determine whether the increase in cell death caused a regenerative response, livers were stained for the proliferative marker Ki67. Notch2-cKO livers contained an increased number of Ki67-positive nuclei compared to control livers (Figure 4C, D). Proliferation was found both in hepatocytes, cholangiocytes, and non-parenchymal cells. All D42 Notch2-cKO mice examined (n=8) were still severely icteric.

Figure 4 Liver architecture and proliferation in D42 Notch2-cKO and control mice
Panel A: H&E-stained image of D42 Notch2-cKO liver with two necrotic areas (n). Notice the surrounding inflammatory cells (arrow) p: portal vein. Panel B: liver/body-weight ratio at D21, D42 and D180 of Notch2-cKOs (black bars) and control mice (white bars; *: p<0.05). Panels C, D: Ki67 expression in D42 control and Notch2-cKO liver, respectively, in comparable peripheral areas. Notice increased amount of Ki67 positive nuclei in Notch2-cKO liver (parenchymal cells: large nucle and non-parenchymal cells: small nucle). Scale bar in panel A is applicable to panels C and D.
The newly formed tubular structures resemble the atypical ductular reaction, with some ducts acquiring a communication with the extrahepatic bile ducts. Unexpectedly, some of the surviving Notch2-cKO mice were no longer icteric when sacrificed at or after 6 months (n=5), whereas severe icterus persisted in others (n=11). All Notch2-cKO livers at this age contained CK19-positive tubular and ductal plate-like structures, but their structural organization varied considerably from liver to liver. Some knockout livers contained mostly disorganized ductal plate-like structures that extended away from the portal tracts (Figure 5B), whereas others contained dilated ducts (Figure 5C-E) that sometimes formed tangles (Figure 5D). These features resemble the atypical ductular reaction that is associated with longstanding hepatic biliary obstruction (23-25).

On inspection, all D180+ Notch2-cKO livers were pale and stiff. Histological examination showed continued development of necrotic areas in icteric mice, while picro-Sirius red staining revealed severe fibrosis (Figure 6B and C) in both the icteric and non-icteric mice. Furthermore, regenerative nodules (adenomas) with only sparse collagen deposition were seen in some icteric livers (Figure 6C and supplemental Figure S2). These features are characteristic for cholestatic fibrosis. The chronic nature of the cholestasis was also evident from the pathology seen in the kidney (supplemental Figure S3).

Figure 5 CK19 expression in D180 livers of Notch2-cKO and control mice
Panel A: D180 control liver with peripheral portal veins mostly associated with a single CK19+ bile duct. Panel B, C: livers of two icteric Notch2-cKO mice (N2KO) with highly irregular CK19+ strands near portal veins, but also extending deep into the parenchyma (B). Higher magnification (inset): CK-19+ structures with and without lumina. Panel C: aberrantly shaped bile ducts around portal veins. Panel D, E: livers of two non-icteric Notch2-cKO (N2KO-NI) mice with a cluster of dilated bile ducts with (D) or without tangles (E). Scale bar in panel A is applicable to panels B and C.
Since ~30% of the 6-month old Notch2-cKO mice were no longer icteric, we assessed the functionality of their newly formed bile duct system. Plasma bilirubin and bile-salt levels in Notch2-cKO mice varied from non-detectable to ~180 µM (total bilirubin) and 6 mM (total bile salts), respectively, in agreement with our observation that Notch2-cKO mice of this age could be both non-icteric and icteric (Figure 6D). The hyperbilirubinemia was of the conjugated type as expected from an obstructive cholestasis (supplemental Figure S4a). Bile production varied widely among Notch2-cKO mice and could not be accurately measured. However, if bile was produced, it contained a high concentration of bilirubin conjugates and bile salts (Figure 6D). High plasma bilirubin levels paralleled high plasma bile-salt levels ($R^2 = 0.7$, supplemental Figure S4b). These data demonstrate that Notch2-cKO mice that remained icteric did not develop a draining bile duct system. We observed mice at 1 year of age and older that had remained icteric and did not excrete bilirubin conjugates or bile salts via the bile duct, demonstrating that a functional bile duct system never developed in a subset of Notch2-cKO mice.

Figure 6 Biliary cirrhosis, and bilirubin and bile salt levels in plasma and bile of D180 Notch2-cKO and control mice
Panels A-C: Sirius red-stained sections of D180 control (A) and two Notch2-cKO (B, C) livers. Notch2-cKO livers show extensive collagen deposition, with “bridging” fibrosis between vessels (arrows). Panel C: two adenomas (a) surrounded by a capsule. Scale bar in panel A is applicable to panels B and C. Panel D: plasma and biliary bilirubin levels in Notch2-cKO and control mice. Note high plasma bilirubin and bile-salt levels in icteric Notch2-cKO mice (N2KO) and normalized levels in non-icteric Notch2-cKO mice (N2KO-NI). These differences were less pronounced in bile.
Results

Figure S2 Liver Histology of 1-year old Notch2-cKO mouse with adenoma
Panels a and b: sections stained with H&E and PAS to show adenoma (ad) that is identifiable by a “distension” line (black asterisks) and the absence of glycogen. Panels c and d: The expression of CarbamoylPhosphate Synthetase (CPS; panel c) was lost in some adenomas, whereas that of GS was lost in others (panel d). p: portal vein, c: central vein.

Figure S3 Renal signs of chronic cholestasis
Panels a and c: D180 control (a) and Notch2-cKO (b) kidney stained with H&E reveal degenerative glomeruli (arrowheads) and cyst formation (asterisks) in Notch2-cKO mice.

Chapter 6 Notch2 is required for cholangiocyte differentiation
To definitively demonstrate that non-icteric Notch2-cKO mice had developed a patent bile duct system with a connection to the extrahepatic bile duct, we injected Mercox resin into the cannulated gall bladder. The mice that remained icteric had, as expected, no connected intrahepatic bile ducts and, at best, some bead-like structures in the liver hilum (Figure 7B). However, Notch2-cKO mice that were no longer icteric produced bile and allowed the production of a cast of their newly formed, small bile duct tree (Figure 7C). Based on the size of these casts (n=3), we estimate that bile drainage from ~15% of the liver suffices to resolve the icterus.

Figure S4 Hyperbilirubinemia in Notch2-cKOs correlates with high conjugated bile-salt levels
Panel a: bilirubin-mono-glucuronide (BMG) and di-glucuronide (BDG), unconjugated bilirubin (UCB), and total bilirubin (TB) in plasma of icteric Notch2-cKOs (n=11). Total bilirubin in control plasma was <5 µmol/L (Fig. 6E). Panel b: high plasma bilirubin levels correlated with high plasma bile salt levels in Notch2-cKOs (R² 0.7; p<0.001).

Figure 7 3D continuity of the biliary tree in D180 livers of Notch2-cKO and control mice
From right to left, 3D continuity of proximal bile duct tree of D180 control (A), icteric Notch2-cKO (B) and no-longer icteric Notch2-cKO (C) mice after retrograde injection of Mercox® via the gallbladder. Note the small biliary tree in no-longer icteric Notch2-cKO mouse (arrows: dilated cannulated bile duct). In icteric Notch2-cKO mouse, liquid Mercox only accumulated in dilated tangles near liver hilum.
Early Notch2 ablation leads to altered expression of biliary transcription factors

Notch2 mRNA expression was permanently decreased in Notch2-cKOs, its levels varying from ~50% of controls at ED16.5 to ~25% of controls after birth (Figure 8). These percentages correspond with the contribution of non-parenchymal (and, hence, non-recombined) liver cells to the liver and the decline of this population with age (26). Hnf6, a key regulator of cholangiocyte differentiation (27, 28), was downregulated to a similar extent as Notch2 in Notch2-cKO livers (Figure 8), implying that the expression of Hnf6 depends quantitatively on that of Notch2. Importantly, no difference in Notch2 and Hnf6 expression was found between D180 icteric and non-icteric Notch2-cKO mice.

The mRNA expression of both Hhex, one of the earliest transcription factors expressed in the liver bud and later involved in bile duct morphogenesis (29), and Sox9, the earliest bile duct-specific transcription factor and involved in the timing of ductal plate remodeling (30), was unaffected by Notch2 deficiency before birth, but at ND1 their mRNA levels were significantly downregulated in Notch2-cKOs, indicating that their expression at this stage was also dependent on Notch2 signaling. Thereafter, however, the abundance of Hhex and Sox9 mRNAs no longer correlated with Notch2 expression. Hhex mRNA levels normalized at D21, increased at D42 and became significantly upregulated in D180 icteric Notch2-cKOs, while Sox9 levels were already significantly upregulated at D21 in such mice (Figure 8). Interestingly and intriguingly, Hhex and Sox9 mRNA levels in D180 Notch2-cKO mice that were no longer icteric, had normalized to control levels (Figure 8). Since Notch2-cKO mice are cholestatic and since Hhex is a target of the bile-acid receptor Farnesoid X Receptor (Fxr) (31), we also investigated the mRNA expression of Fxr and its other well-established target Shp (32, 33). Figure 8 shows that Fxr expression was not affected by Notch2 deficiency, but that Shp expression was elevated in Notch2-cKO mice at D21 and D42, but no longer at D180, irrespective of whether the animal was icteric or not.
The expression of Hnf1β, a well-established downstream target of Hnf6 (34, 35), initially followed the expression pattern of Hnf6 (on ED16.5 and D1), but was no longer suppressed at and after D21 (Figure 8). Foxa1 and Foxa2, which are involved in early liver specification and, thereafter, in the regulation of cholangiocyte proliferation (14, 36), were like Hhex and Sox9, unaffected by Notch2 deficiency before birth, but had become significantly downregulated at ND1 and D21. However, from D21 onwards, the mRNA abundance of Foxa1 no longer correlated with Notch2 expression. Notch-pathway members Jagged1 and Notch3 became significantly upregulated in knockout livers at and after D42 (Figure 8), but the expression of other Notch-associated genes, such as Notch1, Notch4, Dlk1 and Hes1, were not different between Notch2-cKO and control liver (supplemental Figure S5). Neither Hnf1β, Foxa1, Foxa2, Jagged1, nor Notch3 differed in mRNA level between icteric and non-icteric D180 Notch2-cKO mice (not shown).

Figure 8 Expression of biliary transcription factors in Notch2-cKO and control mice
Hepatic mRNA levels of Notch2, Hnf6, Hhex, Sox9, Fxr, Shp, Hnf1β, Foxa1, Foxa2, Jagged1 and Notch3, at ED16.5, D1, D21, D42 and D180 in Notch2-cKO mice (black bars) and their littermate controls (white bars). Values are depicted as mean ± SEM. (*: p≤0.05; n=4-8 per group).
**Results**

*Tgfβ1* and *TgfβR2* mRNA levels (Figure S5 in supplemental material) were significantly upregulated in *Notch2*-cKO livers from D21 onwards, but it should be noted that *Tgfβ* signaling is involved in both bile duct morphogenesis (28) and fibrogenesis (37, 38). The expression of the bile duct markers *Ck19* and *Itgb4*, as hepatocyte differentiation genes *Cebpα*, *Hnf4α* and *Sox17* was also investigated (supplemental Figure S5), but did not differ in a consistent way between *Notch2*-cKO and control livers.

![Graphs showing mRNA expression levels of various genes at different time points.](image)

Figure S5 Expression of biliary transcription factors in Notch2-cKO and control mice (2)

Hepatic mRNA levels of *Notch1*, *Notch4*, *Tgfβ1*, *TgfβR2*, *Hes1*, *Dlk1*, *Hnf4α*, *Ck19*, *Integrinβ4* (*Cd104*), *Sox17* and *Cebpα* at ED16.5, ND1, D21, D42 and D180 in Notch2-cKO mice (black bars) and their littermate controls (white bars). Values are depicted as mean ± SEM. (*: p≤0.05), (n=4-8 animals per group).
Notch2 is indispensable for cholangiocyte differentiation

In the present study, we show that elimination of Notch2 from mouse hepatoblasts before hepatocyte-cholangiocyte differentiation results in the complete absence of ductal plate formation in fetal and perinatal liver. Our findings, therefore, show for the first time that Notch2 determines cholangiocyte cell fate. Notch2\textsuperscript{fl/fl}/AlfpCre mice have normal extrahepatic bile ducts and gallbladders, consistent with the hepatocyte-specific expression of Alfp-Cre (13). The Alfp-Cre transgene, therefore, mediated complete deletion of Notch2 in the future cholangiocyte lineage before ED16, that is, at a relatively early age compared to previous reports (6, 36), where Cre recombinase activity was maximally active around ED16.5 (6). The observed differences in peak Alfp-Cre activity may be due to differential accessibility of different loxP-flanked loci (36).

Two earlier studies were carried out with Notch2\textsuperscript{fl/fl}/Alb-Cre mice (10, 11), which resulted in the perinatal ablation of the Notch2\textsuperscript{fl/fl} alleles (12, 39), that is, after hepatocyte-cholangiocyte differentiation. Notch2\textsuperscript{fl/fl}/Alb-Cre mice show a comparable, but less severe postnatal phenotype than our Notch2\textsuperscript{fl/fl}/Alfp-Cre mice, with a decreased number of bile ducts perinatally and extensive cholangiocyte proliferation during later stages (10, 11). However, in contrast to Notch2\textsuperscript{fl/fl}/Alfp-Cre mice, which do not form a ductal plate prenatally and suffer from a total absence of bile ducts at ND1, Notch2\textsuperscript{fl/fl}/Alb-Cre mice do show embryonic (11) and neonatal (10, 11) ductal plate structures that had yet to form tubular structures at D7 (11) and that had formed multiple, irregular tubular structures at D20. Like Notch2\textsuperscript{fl/fl}/Alfp-Cre mice, Notch2\textsuperscript{fl/fl}/Alb-Cre mice suffered from fibrosis at D120 (10). Interestingly, therefore, the hepatic phenotype of adult Notch2\textsuperscript{fl/fl}/Alb-Cre mice does not differ greatly from that of Notch2\textsuperscript{fl/fl}/Alfp-Cre mice, except that a ductal plate was present in perinatal Notch2\textsuperscript{fl/fl}/Alb-Cre mice, while absent in Notch2\textsuperscript{fl/fl}/Alfp-Cre mice. We defined the presence or absence of a ductal plate by the prenatal expression of CK19 and annexinIV (17) in the portal limiting plate, whereas Lozier \textit{et al.} (10) and Geisler \textit{et al.} (11) used CK19 and Dolichos Biflorus Agglutinin, that is, similar markers. Our findings therefore show that prenatal development of the ductal plate is not necessary for postweaning appearance of bile ductular cells and the formation of tubular structures.

A third study investigated the effects of early ablation of the Notch transcriptional co-factor Rbp-j in Rbp-j\textsuperscript{fl/fl}/Foa3-Cre and Rbp-j\textsuperscript{fl/fl}/Alfp-Cre mice, which resulted in a liver phenotype with a reduction in the number of mature bile ducts at ND1 in both mouse lines (6). Rbp-j\textsuperscript{fl/fl}/Foa3-Cre mice had a more severe phenotype than Rbp-j\textsuperscript{fl/fl}/Alfp-Cre mice, probably due to less development of ductal plate cells embryonically. Based on these data, Zong \textit{et al.} (6) concluded that the Notch pathway, via Rbp-j, regulates both biliary cell fate and bile duct morphogenesis. In conjunction with our data, the findings in Rbp-j-deficient mice suggest that part of the Notch2-mediated effects on bile duct formation proceed in an Rbp-j-independent manner.
The absence of perinatal bile duct formation due to Notch2 deficiency permits secondary bile duct formation after weaning that resembles the atypical ductular reaction

Despite the complete lack of bile ducts and the accompanying severe cholestasis, ~85% of Notch2-cKOs survived to adulthood, in all likelihood because of the hydrophilic character of rodent bile salts (40). The scattered CK19-positive cells that appeared in the vicinity of large portal veins in D21 Notch2-cKO livers resembled the appearance of CK19-positive cells during prenatal ductal-plate formation. Postweaning bile duct formation in Notch2-cKO livers proceeded at a slow pace, with the appearance of CK19-positive ductal plate-like structures and tubules in the vicinity of large portal veins at 6 weeks and the disappearance of jaundice in ~30% of affected mice at 6 months of age. The sprouting cell strands and aberrantly-shaped, proliferating tubules, both in the vicinity of portal veins and deeper into the periportal parenchyme of Notch2-cKO livers are strikingly reminiscent of the atypical (type 2) ductular reaction that is seen in response to many types of hepatic injury in rodents (23, 25) and after massive hepatic necrosis, longstanding hepatic biliary obstruction and chronic cholestatic liver diseases in humans (22-24). The extent to which a three-dimensional network of the bile ducts had formed and connected to the extra-hepatic bile ducts, and the bilirubin and bile-salt excretion into the bile appeared to correspond with the varying degrees of morphological development of the ductular structures (Figures 5 and 6). Although a functional excretory system only developed in ~30% of the Notch2-cKO livers, our observations do show that atypical bile duct formation does allow the (re)establishment of a biliary system.

The origin of the cells in the periportal region that produce the cholangiocytes that are responsible for the secondary bile duct formation is not clear at present. Neo-formed bile ducts in atypical ductular reaction are thought to originate either from transdifferentiation of hepatocytes into cholangiocytes or from oval (stem) cells, which in rodents occurs in association with oval-cell proliferation (23, 25). A stem-cell origin of the cholangiocytes in Notch2-cKO livers, with postnatally an ab-initio onset of the “normal” pathway of cholangiocyte differentiation, would only be possible if the Alfp promoter-induced activation of Cre expression does not occur. Instead, the finding that neither Notch2 nor Hnf6 mRNA levels were restored in the liver of no-longer icteric Notch2-cKOs strongly suggests that the neo-formed bile ducts do not develop from Notch2-positive cells that have escaped recombination.
Notch2 deficiency permanently downregulates Hnf6, but downregulates other biliary differentiation genes only perinatally

The expression of Hnf6, a key regulator of cholangiocyte differentiation (34, 35), was significantly and permanently downregulated in Notch2-cKO livers, implying that Hnf6 expression is dependent on Notch2, thus lies downstream of Notch2 in the cholangiocyte differentiation cascade. Although our expression data contrast with the continued presence of HNF6 protein in perinatal Notch2-fl/fl/Alb-Cre mice (11), they are consistent with the only transient perinatal downregulation of Hnf1β mRNA expression in Hnf6-knockout mice (27). Furthermore, Rbpj-fl/fl/Hnf6-fl/fl/Alb-Cre double knockout mice were recently shown to have a more severe phenotype than Rbpj-fl/fl/Alb-Cre mice (41), but nevertheless developed ductal plate structures at ED16.5, unlike our Notch2-fl/fl/Alfp-Cre mice. Although the authors propose a parallel action of Hnf6 and Notch during biliary differentiation, Albumin-Cre mediated excision of Hnf6 alone did not affect intrahepatic bile duct morphology nor expression levels of biliary transcription factors Hnf1β and Sox9. Therefore, our findings, with permanent suppression of Hnf6 mRNA levels at all time-points studied, suggests a role for Notch2 upstream of Hnf6 in the cholangiocyte differentiation cascade.

The forkhead box transcription factors A1 and A2 (Foxa1 and Foxa2) are involved in liver specification and are, during later stages, required for regulating cholangiocyte proliferation (14, 29, 36). Except at D42, Foxa2 mRNA levels were downregulated in postnatal Notch2-cKO livers. Foxa1 expression initially followed the same pattern, but became significantly upregulated during secondary bile duct formation, which suggests that Foxa1 and Foxa2 are also downstream targets of Notch2 during cholangiocyte differentiation and that Foxa1 may be involved in the observed postweaning cholangiocyte proliferation in Notch2-cKO livers (36). Other biliary transcription factors, such as Hhex, Hnf1β, Sox9, and Cebpα, were only downregulated during the perinatal period in Notch2-cKO livers and normalized in expression (Hnf1β, Cebpα) or even became upregulated after weaning (Hhex, Sox9), coincident with the observed secondary bile duct formation. Hnf1β, Sox9, and Cebpα are all established downstream targets of Hnf6 (30, 34, 35). Hhex can alter the expression of Hnf6, Hnf1β and Hnf4α (29). The perinatal downregulation and the subsequent upregulation after weaning of these transcription factors in Notch2-cKO livers suggests that, even though these transcription factors are downstream targets of Notch2 during pre- and perinatal stages, they become activated again by a factor different from Notch2 during secondary bile duct development. It is, therefore, striking that Hhex and Sox9 were significantly upregulated in 6 months old icteric Notch2-cKOs, but no longer in non-icteric Notch2-cKOs of the same age. Hhex expression was recently shown to be regulated by the bile acid-activated transcription factor Fxr during adaptation of hepatocytes to chronic bile-acid exposure (31).
Although the expression of Fxr itself was not changed, expression of its well established target Shp (32, 33) was elevated in Notch2-cKO mice at D21 and D42. Unexpectedly, however, Shp expression was no longer upregulated in both icteric and non-icteric mice at D180, suggesting that bile acids are necessary to initiate, but not to expand secondary bile duct development.

The expression of the Notch pathway members Notch3 and Jagged1 also became significantly upregulated coincident with the onset of secondary bile duct proliferation. Both NOTCH3 and JAGGED1 expression is upregulated in the ductular reactive cells in the liver of patients with Alagille syndrome (42). This finding suggests an important compensatory role for Notch3/Jagged1 in the absence of Notch2 and may explain why the expression of Hes1, a well-known downstream target of Notch signaling, was unaffected by Notch2 deficiency.
Conclusion

Deletion of Notch2 during early liver organogenesis causes complete bile duct agenesis, which resolves the discussion on the role of Notch2 in cholangiocyte cell-fate determination. It is of interest to note that the effects of Notch2 deficiency become less pronounced with later ablation, suggesting that Notch2 controls successive steps in bile duct development. After weaning, cholangiocyte differentiation and bile duct development are resumed in Notch2-cKO livers in a Notch2-independent fashion, with histological features that resemble the atypical ductular reaction. The cholestasis-induced activation of Fxr/Shp might mediate the increase in Hhex expression, which then upregulates Sox9, Foxa1, Cebpα, and possibly also Notch3, and Jagged1 expression. We, therefore, hypothesize that Hhex is responsible for the reinitiation of bile duct development after weaning, using much of the transcriptional cascade that also regulates bile duct development perinatally. The important findings of our study are, therefore, the demonstration that Notch2 occupies the top of the transcriptional cascade controlling prenatal cholangiocyte differentiation and that a Notch2-and Hnf6-independent program of cholangiocyte differentiation is activated in postweaning cholestatic mice that appears to use an otherwise very similar signaling cascade. Possibly, the Fxr-dependent factor Hhex functions at the top of this cascade. The resulting, seemingly unorchestrated proliferation of bile ducts can form a functional network in ~30% of cases. We further hypothesize that our observations resemble the development of a rudimentary bile duct tree in patients with biliary atresia that have undergone a hepatoportoenterostomy (Kasai procedure).
Chapter 6 *Notch2* is required for cholangiocyte differentiation
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Reference list


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