Liver-Specific Inactivation of *Notch2*, but not *Notch1*, Compromises Intrahepatic Bile Duct Development in Mice

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The Notch pathway is an evolutionary conserved, intercellular signaling pathway that plays an important role in cell fate specification and the embryonic development of many organs, including the liver. In humans, mutations in the Notch receptor ligand *Jagged1* gene result in defective intrahepatic bile duct (IHBD) development in Alagille syndrome. Developmental abnormalities of IHBD in mice doubly heterozygous for *Jagged1* and *Notch2* mutations propose that interactions of Jagged1 and its receptor Notch2 are crucial for normal IHBD development. Because different cell types in the liver are involved in IHBD development and morphogenesis, the cell-specific role of Notch signaling is not entirely understood. We investigated the effect of combined or single targeted disruption of *Notch1* and *Notch2* specifically in hepatoblasts and hepatoblast-derived lineage cells on liver development using *AlbCre* transgenic mice. Hepatocyte differentiation and homeostasis were not impaired in mice after combined deletion of *Notch1* and *Notch2* (*N1N2*$^{F/F}$*AlbCre*). However, we detected irregular ductal plate structures in *N1N2*$^{F/F}$*AlbCre* newborns, and further postnatal development of IHBD was severely impaired characterized by disorganized ductular structures accompanied by portal inflammation, portal fibrosis, and foci of hepatocyte feathery degeneration in adulthood. Further characterization of mutant mice with single deletion of *Notch1* (*N1*$^{F/F}$*AlbCre*) or *Notch2* (*N2*$^{F/F}$*AlbCre*) showed that *Notch2* but not *Notch1* is indispensable for normal perinatal and postnatal IHBD development. Further reduction of *Notch2* gene dosage in *Notch2* conditional/mutant (*N2*$^{F/LacZ}$*AlbCre*) animals further enhanced IHBD abnormalities and concomitant liver pathology. *Conclusion:* Notch2 is required for proper IHBD development and morphogenesis. (HEPATOLOGY 2008;48:607-616.)

See Editorial on Page 358

In humans and rodents, intrahepatic bile duct (IHBD) development begins with the condensation of hepatoblasts forming a single continuous cell layer around the larger portal veins called the ductal plate. Later, parts of the ductal plate reduplicate and dilate to form tubular structures that are subsequently incorporated in the portal mesenchyme. The remaining nontubular single-layered cells of the ductal plate are eliminated via apoptosis while the tubular structures further undergo a branching process to form the biliary tree. This process of ductal plate remodeling starts at the portal vein at ap-

Abbreviations: AGS, Alagille syndrome; IFN-α, interferon-α; IHBD, intrahepatic bile duct; P, postnatal day; WT, wild-type; X-gal, X-galactosidase.

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proximately embryonic week 8 and embryonic day 16.5 in humans and mice, respectively, progresses toward the periphery of the liver, and continues for the first weeks after birth.[1-3] In humans, abnormalities in physiological ductal plate remodeling can lead to a variety of diseases called ductal plate malformation, such as congenital hepatic fibrosis or Caroli's syndrome. Other congenital disorders of IHBD include Alagille syndrome (AGS), which is caused by mutations in the *Jagged1* gene encoding the Notch ligand Jagged1.[4,5] AGS is a multisystem disorder characterized by developmental abnormalities of the heart, eye, skeleton, and liver. Though progressive loss of interlobular bile ducts is the typical finding in liver biopsies,[6,7] bile duct proliferation may also be observed early in the course of AGS.[8,9]

The Notch signaling pathway plays an important role in cell fate specification and the embryonic development of many organs, including the hepatobiliary system. In mammals, four transmembrane Notch receptors (Notch1-4) and five ligands, including Dll1, Dll3, Dll4, Jagged1, and Jagged2, have been described.[10] Notch signaling activation is initiated by γ-secretase–dependent cleavage and release of cytoplasmic Notch-IC after ligand-receptor binding on neighboring cells. After translocation to the nucleus, Notch-IC binds and converts RBP-Jκ from a transcriptional repressor into an activator leading to transcription of Notch target genes such as *Hes* and *Hey* family genes. Expression analyses in human and mouse liver tissues have found Notch receptors and ligands to be expressed in embryonic and adult livers.[11-14] Whereas mice homozygous for null mutations in Notch pathway genes such as *Notch1*, *Notch2*, *Jagged1*, *Dll1*, or *Rbpj* could not be studied for proper organ development and homeostasis due to early embryonic lethal phenotypes,[10] recent studies using conditional inducible and developmental mouse models have shed light on the role of single Notch receptors and ligands in the hepatobiliary system. Postnatal inducible inactivation of *Notch1* using *MxCre* mice caused nodular regenerative hyperplasia by regulating hepatic proliferation but no biliary abnormalities.[12] In another study, mice heterozygous for a *Jagged1* null mutation and a hypomorphic *Notch2* allele showed features of human AGS, including bile duct paucity.[14] However, conditional hepatoblast-specific inactivation of *Jagged1* using *AlfpCre* mice had a normal bile duct development, as did the additional implementation of one hypomorphic *Notch2* allele.[15] Bile duct abnormalities were observed in 50% of mice only when a *Jagged1* null allele was introduced in combination with a conditional *Jagged1* allele.[15] An intricate network of different cell types including hepatoblasts, vascular epithelial cells, portal mesenchymal cells, and periportal connective tissue drives IHBD development and morphogenesis.[2,16] Thus, although Jagged1 has an important function during bile duct development, it may not act cell-autonomously in hepatoblasts but in adjacent cells to activate Notch signaling in hepatic progenitor cells and/or other cell compartments that are crucial for proper IHBD development. However, the cell-specific site of action of Notch2 has remained unclear and a possible contribution of other Notch receptors in tissue-specific knockout models has not been investigated. We investigated the effect of combined or single conditional ablation of *Notch1* and *Notch2* in hepatobiliary development and homeostasis using *AlbCre* mice and demonstrate that *Notch2* but not *Notch1* in hepatoblasts and hepatoblast-derived lineage cells is essential for normal IHBD development and morphogenesis in mice.

Materials and Methods

Mice. Mice carrying conditional knockout alleles for *Notch1* (*floxed Notch1, N1*$^{F/F}$ mice)[17] and *Notch2* (*floxed Notch2, N2*$^{F/F}$ mice)[18] were crossed with transgenic mice carrying a *Cre* gene under control of the albumin enhancer promoter (*AlbCre* mice).[19] After multiple rounds of crossing, we obtained the following genotypes that were used in this study: *N1N2*$^{F/F}$*AlbCre, N1*$^{F/F}$*N2*$^{F/+}$*AlbCre, N1*$^{F/+}$*N2*$^{F/F}$*AlbCre, N1*$^{F/F}$*AlbCre*, and *N2*$^{F/F}$*AlbCre*. For breeding of conditional *Notch1/Notch2* double-knockout animals, male *N1N2*$^{F/F}$*AlbCre* mice were mated with female *N1N2*$^{F/F}$ mice. All strains were maintained on a C57Bl6/Sv129 background. In all experiments *AlbCre*-negative littermates served as a control unless stated otherwise. Heterozygous *Rosa26-β-gal* reporter mice[20] were used to detect Cre-induced recombination events. For Notch1 expression studies, we used transgenic *Notch1-GFP* (*N1-GFP*) reporter mice[21]; for Notch2 expression studies, heterozygous mutant *Notch2* mice were used (*N2*$^{+/LacZ}$ mice, previously referred to by Hamada et al.[22] as *Notch2*$^{+/m}$). In these mice, 5 of the 6 ankyrin repeats and part of the downstream sequence of the *Notch2* gene are replaced with the *LacZ* gene.[22] For *Notch2* gene dosage studies, these mice were also used to create *Notch2* conditional/mutant mice (*N2*$^{F/LacZ}$*AlbCre*). Genotyping was performed via polymerase chain reaction or X-galactosidase (X-gal) staining of tails in heterozygous *Notch2* mutant mice (sequences shown in Supplementary Table 1). Mice were handled according to protocols that follow national guidelines for ethical animal treatment, and all experiments were performed according to the protocols approved by our Institutional Animal Care and veterinarian office.

Hepatocyte Isolation. Hepatocytes were isolated from 8-week-old animals by a standard in situ two-step

retrograde collagenase-perfusion technique (Liberase-Blendzyme-3, Roche, Germany) as described.[23] Seventy percent partial hepatectomy in 8-week-old C57Bl6 mice was performed as described.[24]

Protein Isolation and Western Blot Analysis. For preparation of whole-cell protein extracts, livers or primary hepatocytes were homogenized in Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and protease- and phosphatase-inhibitor cocktail). The lysate was gently sonicated and clarified by centrifugation (14,000 rpm for 10 minutes at 4°C), snap-frozen in liquid nitrogen, and stored at −80°C until assayed. Protein extracts were analyzed via discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described.[23] Antibodies and conditions used are listed in Supplementary Table 2.

Histology and Immunohistochemical Analysis. For histological analysis, livers were removed, fixed in 4% neutral phosphate-buffered paraformaldehyde for 16 hours, embedded in paraffin, and sectioned. Serial 3.5-μm-thick sections were stained with hematoxylin-eosin or Sirius red using a standard protocol. Masson's trichrome staining was performed using a trichrome stain kit (Sigma, Germany). For immunohistochemical analyses and X-gal staining on frozen sections, tissues were processed as described[25] using antibodies as shown in Supplementary Table 2. For detection of mouse antibodies, a MOM kit (Vector Laboratories, UK) was used to block unspecific binding.

Results

Targeted Liver-Specific Disruption of Notch1 and Notch2. Constitutive knockout mice for *Notch1* or *Notch2* display embryonic lethality before embryonic day 11.5.[22,26] To study the function of Notch signaling in perinatal and postnatal liver development and homeostasis, we generated conditional knockout mice in which both *Notch1* and *Notch2* were inactivated, specifically in the liver (*N1N2*$^{F/F}$*AlbCre* mice). In the adult liver, albumin is expressed exclusively in hepatocytes. Consequently, Notch1 and Notch2 protein were not detectable in hepatocytes isolated from 8-week-old *N1N2*$^{F/F}$*AlbCre* mice (Fig. 1A). However, in the embryonic liver, albumin expression occurs in hepatoblasts as early as 13.5 days of gestation before intrahepatic bile ducts begin to differentiate from periportal hepatoblasts. In mice, the process of bile duct development and morphogenesis starts at around embryonic day 15 and extends until the first 2 weeks of age.[2] Thus, recombination of *floxed* alleles in mice carrying the *AlbCre* transgene can also be found in intrahepatic bile ducts in the adult mouse.[27,28] Consistently, when crossing *AlbCre* mice with a *Rosa26* reporter mouse,[20] liver parenchymal cells and the vast majority of bile ducts but not hematopoetic cells or portal vein mesenchyme were X-gal–positive when analyzed at postnatal day (P) 1 and P30, respectively (Fig. 1B). Because mature bile duct epithelial cells do not express albumin,[2] these data confirm that in *AlbCre* transgenic mice Cre expression occurs in hepatoblasts and/or precursors of intrahepatic bile duct cells before termination of bile duct development.

Liver-specific conditional double-mutant *N1N2*$^{F/F}$-*AlbCre* mice were born at Mendelian frequencies without apparent abnormalities. Because IHBD development in the mouse continues beyond the first weeks after birth we first analyzed the histological organization of the liver architecture after conclusion of postnatal bile duct development in 4-week-old *N1N2*$^{F/F}$*AlbCre* mice and control littermates. Livers of 4-week-old *N1N2*$^{F/F}$*AlbCre* mice were not distinguishable from controls on gross examination. For histological analysis we performed hematoxylin-eosin staining and pan-CK staining to identify the intrahepatic bile duct status in mutant and control mice.[2,29] *N1N2*$^{F/F}$ control mice had normal liver architecture and bile duct morphology (Fig. 2A,B). In contrast, combined deletion of *Notch1* and *Notch2* resulted in a disorganized biliary system. In all mice investigated at the age of 4 weeks (n = 12), portal and periportal areas and interlobular septs displayed multiple arborizing pan-CK–positive ductular structures that extended far into the hepatic lobe (Fig. 2C-F). Mature differentiated bile ducts, integrated into the portal mesenchyme, could be observed only in the hilar regions of the liver lobes around large portal veins (data not shown). In addition to these irregular ductular structures, which were abundant in all *N1N2*$^{F/F}$*AlbCre* mice analyzed, portal areas with proliferation and distortion of mature bile ducts accompanied by mild portal inflammation as assessed with anti-CD45 staining were also observed in 9 of 12 animals (Fig. 2G,H). Trichrome staining highlights enlarged portal tract expansion with mild deposits of collagen (Fig. 2I). These morphological changes were most pronounced in the periphery of the hepatic lobes and are suggestive of local cholestasis. In this context, small foci of hepatocyte feathery degeneration (bile infarcts) were also observed in 5 of 12 animals (Fig. 2J).

Early Postnatal IHBD Development Is Impaired in N1N2$^{F/F}$AlbCre Mice. Morphological findings in 4-week-old *N1N2*$^{F/F}$*AlbCre* mice suggest abnormal development of IHBD. To detect early differences in IHBD differentiation and morphogenesis between control and *N1N2*$^{F/F}$*AlbCre* mice, we analyzed mice at P1, P10, and

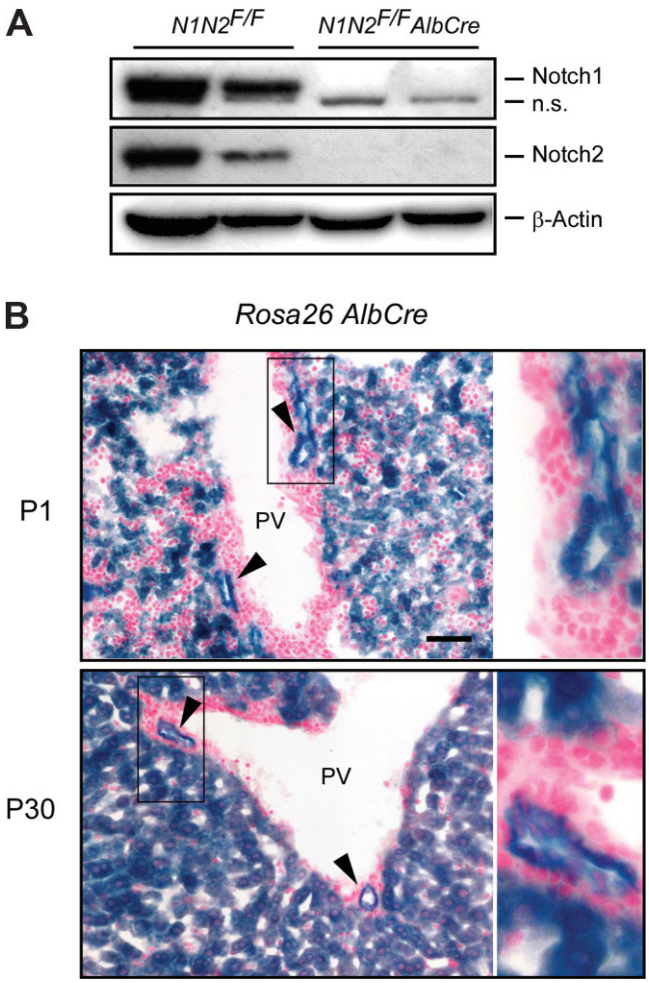

Fig. 1. Targeted liver-specific disruption of *Notch1* and *Notch2*. (A) Protein lysates were prepared from primary hepatocytes isolated from 8-week-old *N1N2*$^{F/F}$ mice and *N1N2*$^{F/F}$*AlbCre* littermates and subjected to western blot analysis using anti-Notch1, anti-Notch2, and anti-β-actin antibodies. n.s., nonspecific band. (B) Cre-induced recombination of *floxed* alleles occurs in both hepatocytes and biliary epithelial cells in livers from *Rosa26AlbCre* reporter animals at P1 and P30 as assessed with X-gal staining. Arrowheads indicate bile ducts. The outlined areas are magnified in the right panels. PV, portal vein. Scale bar = 50 μm.

P20. In control mice, typical ductal plate remodeling at P1 was apparent from the detection of pan-CK–positive epithelial cells forming tubular and nontubular structures around the larger portal veins (Fig. 3A). At P10 (Fig. 3C) and P20 (Fig. 3E), the tubular structures progressed further into mature differentiated bile ducts well integrated into the portal mesenchyme, whereas the nontubular part was largely eliminated, displaying only few pan-CK–positive ductal plate remnants. Ductal plate cells were also detected in *N1N2*$^{F/F}$*AlbCre* animals at P1; however, in contrast to control animals, these pan-CK–positive cells were mostly arranged irregularly around the portal veins and very rarely formed typical tubular structures (Fig. 3B). At P10 (Fig. 3D), the vast majority of portal tracts did not contain differentiated bile ducts. Instead, ductal plate remnants and abnormal CK-positive epithelial cells were abundant in the periportal area. Moreover, in 3 of 6 animals analyzed at P10, we observed small foci of feath-

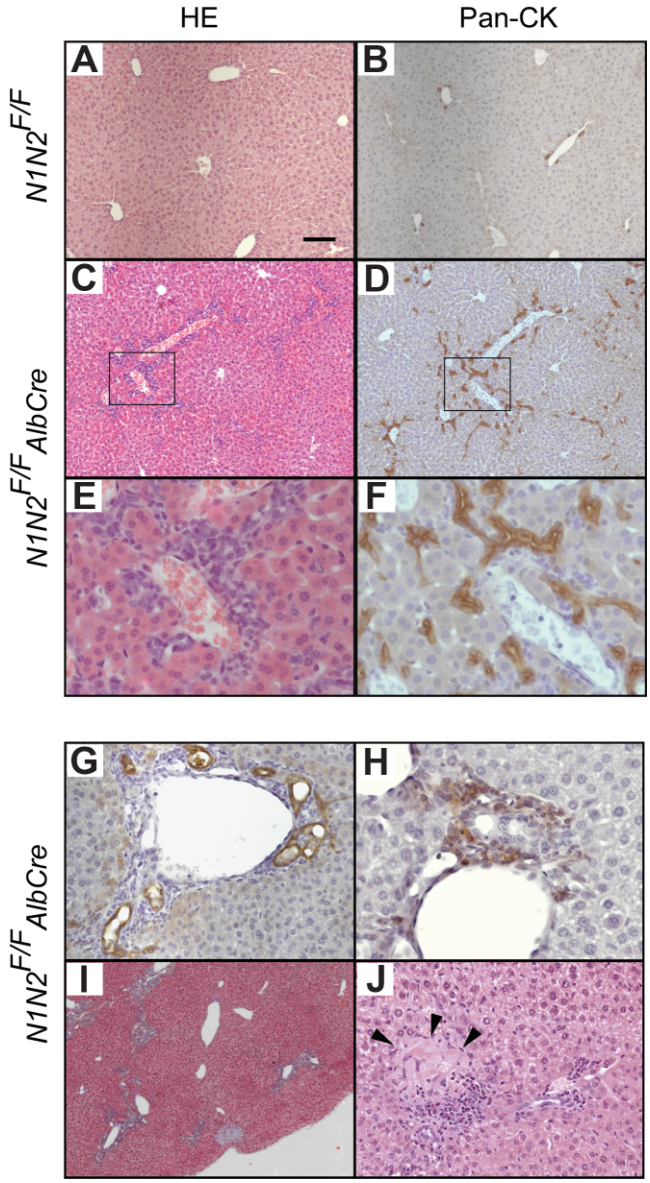

Fig. 2. Combined liver-specific disruption of *Notch1* and *Notch2* results in abnormal IHBD status. (A,B) Hematoxylin-eosin staining and pan-CK immunostaining of bile ducts of control livers at 4 weeks of age reveal normal parenchymal and portal tract architecture. (C-F) Serial sections of mutant livers display a disorganized biliary system characterized by multiple arborizing pan-CK–positive tubular structures. Insets in panels C and D are amplified in panels E and F. (G) Increased number of dilated and distorted bile ducts frequently surround larger portal veins as assessed with pan-CK staining. (H) Anti-CD45 immunostaining reveals periportal leukocyte infiltration. (I) Trichrome staining at low magnification demonstrates portal tract expansion with mild periportal and interlobular deposits of collagen. (J) Hematoxylin-eosin staining reveals a small focus of feathery hepatocyte degeneration (bile infarct, arrowheads). Scale bar in panel A = (E,F,H) 25, (G,J) 50, (A-D) 100, and (I) 200 μm.

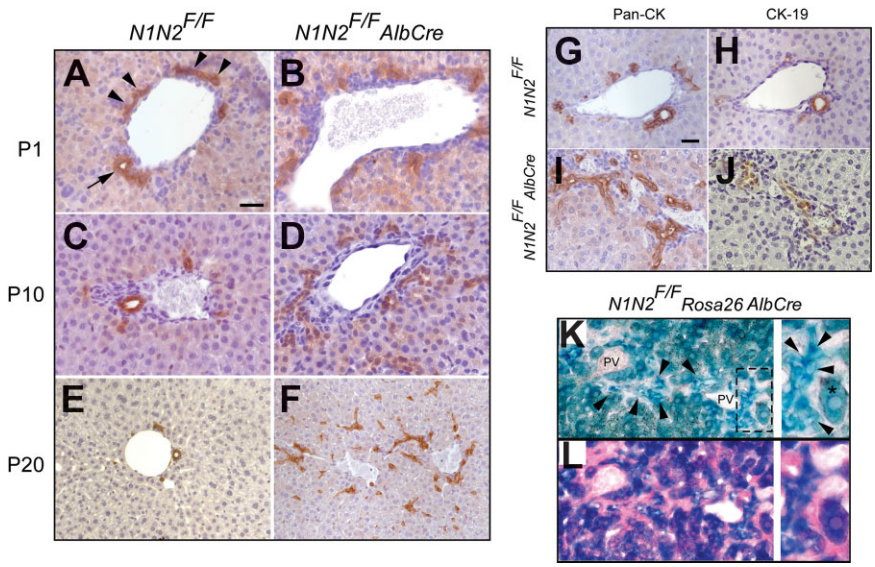

Fig. 3. Early postnatal IHBD development is impaired in *N1N2*$^{F/F}$*AlbCre* mice. Bile duct status in livers from (A,C,E) control and (B,D,F) *N1N2*$^{F/F}$*AlbCre* mice was analyzed at P1, P10, and P20 with pan-CK immunostaining. (A) In control livers, tubular (arrow) and nontubular (arrowheads) ductal plate structures can be observed at P1. The tubular portion further progressed into well-differentiated bile ducts, while the remaining ductal plate cells were progressively eliminated at (C) P10 and (E) P20. (B) In mutants, pan-CK–positive ductal plate cells are arranged irregularly and do not form typical tubular structures around most portal veins at P1. (D) At P10, abundant ductal plate remnants and abnormal pan-CK–positive cells are typically observed in mutant livers. (F) At P20, mutant livers largely lack regular bile ducts but display multiple disorganized ductular structures. (G-J) Adjacent sections of control and mutant livers at P20 were subjected to pan-CK and CK19 immunostaining. (H) In contrast to mature bile ducts in control sections, (J) CK19 staining is weak in irregular ductules of mutant livers. (K,L) X-gal staining of a liver section from a *N1N2*$^{F/F}$*Rosa26AlbCre* mouse at P20 reveals Cre-induced recombination events in both abnormal ductular structures (arrowheads) and hepatocytes (asterisk). The section in panel K was counterstained with nuclear fast red in panel L. The outlined area in panel K is amplified in the right panel. PV, portal vein. Scale bar in panel A = (A-D,K,L) 25 and (E,F) 50 μm. Scale bar in panel G = (G-J) 25 μm.

ery degeneration in the periphery of the liver lobes (data not shown). At P20 (Fig. 3F) the number of these pan-CK–positive epithelial cells further increased now forming strings of cells and tubular structures. However, these structures appeared disorganized and mostly not integrated into the portal mesenchyme.

Biliary epithelial cells become positive for polyclonal pan-CK antibodies early with ductal plate formation, while CK19 expression increases with maturation of bile ducts.[2] In this context, mature bile ducts of P20 control mice stained positive for both pan-CK and CK19 (Fig. 3G,H). In contrast, CK19 staining was weak in the pan-CK–positive duct-like structures observed in *N1N2*$^{F/F}$*AlbCre* animals (Fig. 3I,J). To analyze if these structures in *N1N2*$^{F/F}$*AlbCre* animals arise from cells in which *Notch1* and *Notch2* genes have been targeted by Cre-recombinase, we generated *N1N2*$^{F/F}$*Rosa26AlbCre* reporter mice and found X-gal staining of both hepatocytes and irregular ductular epithelial cells (Fig. 3K,L). Consequently, when analyzing livers from embryonic day 17.5 *N1N2*$^{F/F}$*Rosa26AlbCre* embryos, we detected Cre activity via X-gal staining in approximately 40% to 50% of liver cells (Supplementary Fig. 1A,B). In addition, polymerase chain reaction performed with DNA isolates from embryonic day 17.5 *N1N2*$^{F/F}$*AlbCre* livers using primers specific for deleted *Notch1* and *Notch2* alleles shows Cre-induced recombination of both alleles (Supplementary Fig. 1C).

In summary, cell-specific, combined disruption of *Notch1* and *Notch2* led to impaired IHBD development with the detection of multiple irregular duct-like structures, most likely because of impaired morphogenesis and maturation of the biliary tree.

Notch2, but Not Notch1, Is Indispensable for Normal IHBD Development. To elucidate whether both *Notch1* and *Notch2* are required for normal bile duct development and morphogenesis, we analyzed 4-week-old mutant mice that had at least one wild-type (WT) allele of *Notch1* or *Notch2*, respectively (*N1*$^{F/F}$*AlbCre*, *N1*$^{F/F}$-*N2*$^{F/+}$*AlbCre*, *N1*$^{F/+}$*N2*$^{F/F}$*AlbCre*, and *N2*$^{F/F}$*AlbCre*, [n = 5–8 each]). The phenotype observed in double-mutant *N1N2*$^{F/F}$*AlbCre* animals was completely rescued in mice carrying only one or two WT *Notch2* alleles. Bile duct structures and liver architecture observed in *N1*$^{F/F}$-*N2*$^{F/+}$*AlbCre* or *N1*$^{F/F}$*AlbCre* mice did not differ from *Cre*-negative littermates (Fig. 4A,B). Of note, we did not observe liver hyperplasia in mice lacking *Notch1* as reported for *N1*$^{F/F}$*MxCre* mice after postnatal inactivation of *Notch1*.[12] Neither an increased liver weight/body weight ratio at 4 weeks or 4 months of age nor enhanced

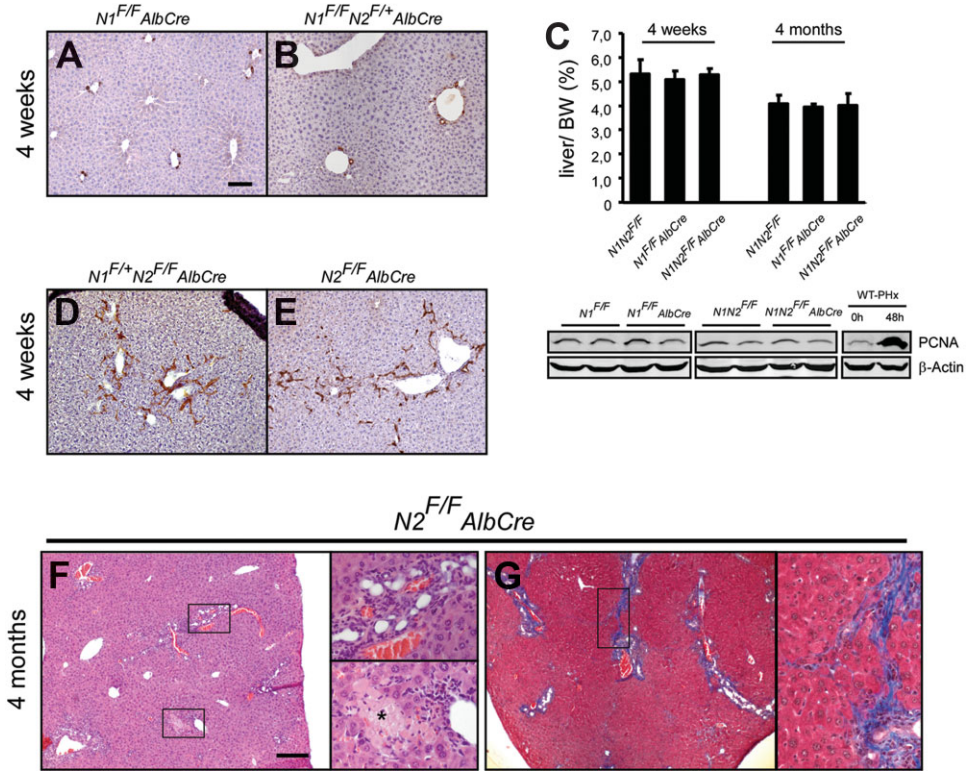

Fig. 4. Single conditional deletion of *Notch2*, but not *Notch1*, is sufficient to impair IHBD development. Normal bile duct status in livers from 4-week-old (A) $N1^{F/F}$*AlbCre* and (B) $N1^{F/F}N2^{F/+}$*AlbCre* mice as assessed with pan-CK immunostaining. (C) Conditional deletion of *Notch1* alone or in combination with *Notch2* did not alter organ size due to enhanced hepatocyte proliferation. Liver weight as a percentage of body weight was determined in mice of indicated genotype and age (upper panel), and western blot analysis of liver lysates from 4-week-old mice using anti–proliferating cell nuclear antigen and anti–β-actin antibodies (lower panel) were performed. Liver lysates from WT mice subjected to partial hepatectomy (PHx) at the time points 0 hours and 48 hours served as a control. Livers from 4-week-old (D) $N1^{F/+}N2^{F/F}$*AlbCre* or (E) $N2^{F/F}$*AlbCre* mice display IHBD abnormalities as observed in $N1N2^{F/F}$*AlbCre* animals as assessed by pan-CK immunostaining. (F) Hematoxylin-eosin staining of a liver section from a 4-month-old $N2^{F/F}$*AlbCre* mouse demonstrates abnormal liver architecture characterized by portal inflammation, fibrosis, bile duct dilation, proliferation (enlarged in right upper panel), and bile infarcts (asterisk in enlarged lower panel). The outlined areas in panel F are amplified in the right panels. (G) Trichrome staining illustrates portal tract expansion with portal and periportal fibrosis. The outlined area is amplified in the right panel. Scale bar in panel A = (A,B,D,E) 100 μm. Scale bar in panel F = (F,G) 200 μm.

proliferation of hepatocytes were detected in $N1^{F/F}$*AlbCre* or $N1N2^{F/F}$*AlbCre* animals as assessed by proliferating cell nuclear antigen western blot analysis (Fig. 4C) or bromodeoxyuridine immunostaining after adding bromodeoxyuridine to the drinking for 7 days (data not shown), indicating that liver-specific disruption of *Notch1* alone or in combination with *Notch2* does not alter organ size by enhanced spontaneous hepatocyte proliferation. When analyzing the bile duct status in livers of mutant mice carrying two *floxed* alleles of *Notch2* but one or two WT alleles of *Notch1* ($N1^{F/+}N2^{F/F}$*AlbCre* or $N2^{F/F}$*AlbCre*, respectively), we found the same morphological phenotype as in double-mutant $N1N2^{F/F}$*AlbCre* mice (Fig. 4D,E). Also, when analyzed at P1, $N2^{F/F}$*AlbCre* displayed irregular ductal plates with very few typical tubular structures (data not shown) indistinguishable from $N1N2^{F/F}$*AlbCre* mice. When analyzing $N2^{F/F}$*AlbCre* animals at 4 months of age, multiple irregularly shaped interlobular bile ducts of varying size frequently not integrated into the portal mesenchyme were the predominant findings. In 3 of 6 animals, these structural biliary abnormalities were accompanied by morphological alterations typically seen as a consequence of cholestasis, such as portal inflammation, bile duct proliferation, portal tract expansion, and portal fibrosis (Fig. 4F,G). These results suggest that Notch1 and Notch2 have nonredundant functions in IHBD development and that defective Notch2 signaling is responsible for structural abnormalities observed in $N1N2^{F/F}$*AlbCre* mice that cannot be compensated upon genetic reconstitution with WT *Notch1*. Of note, technically, we were not able to reliably demonstrate the expression profile of Notch proteins in control and mutant animals via immunohistochemistry using various antibodies under various conditions. Instead, we used transgenic *Notch1-GFP*[21] and heterozygous mutant $Notch2^{+/LacZ}$ reporter mice[22] to analyze hepatic Notch expression profile during IHBD development. Notch1 expression was notably absent in bile ducts but could be

detected in hepatocytes of *Notch1-GFP* mice both at P0 and P50. Notch1 expression was highest in a number of cells with small cytoplasm distributed throughout the liver at P0, most likely cells of the hematopoetic system such as lymphoid cells (Supplementary Fig. 2A-D). In contrast, when analyzing *Notch2*$^{+/LacZ}$ animals via X-gal staining, we found the strongest staining in both developing (Supplementary Fig. 2E) and mature (Supplementary Fig. 2F) bile ducts, whereas less intense staining could be observed in hepatocytes and other liver cells in newborns and in hepatocytes in P50 animals. This expression profile further supports our conclusions deduced from morphological findings in single mutant mice that Notch2, but not Notch1, plays a decisive role in IHBD development.

Severity of Bile Duct Malformations Is Further Enhanced in Notch2 Conditional/Mutant (N2$^{F/LacZ}$AlbCre) Animals. In *AlbCre* animals, Cre-mediated deletion of *floxed* alleles occurs progressively with age[30] and is most likely incomplete in hepatoblasts in *N2*$^{F/F}$*AlbCre* animals during embryogenesis when the first ductal plates form around the large central portal veins (see Supplementary Fig. 1). To test whether further putative reduction in *Notch2* gene dosage in hepatoblasts at earlier stages during embryonic development would further enhance biliary and concomitant structural abnormalities in central parts of the liver, we generated *Notch2* conditional/mutant mice (*N2*$^{F/LacZ}$*AlbCre*) and analyzed livers at 4 weeks of age (n = 9). Whereas livers of heterozygous *Notch2* mutant (*N2*$^{F/LacZ}$) control animals (n = 5) all displayed normal liver architecture and bile duct status (Fig. 5A,B), all *N2*$^{F/LacZ}$*AlbCre* animals showed an IHBD morphology similar to that observed in *N2*$^{F/F}$*AlbCre* mice (Fig. 5C). However, although IHBD abnormalities and concomitant pathology were detected predominantly in the liver periphery in *N2*$^{F/F}$*AlbCre* mice, central parts of the liver in the majority of *N2*$^{F/LacZ}$*AlbCre* animals were severely affected by bile duct abnormalities, characterized by abundant irregular biliary structures within inflamed and enlarged portal tracts (Fig. 5D). We also found large portal tracts with actual bile duct paucity containing only primitive non-remodeled, nontubular ductal plate structures (Fig. 5E). Consequently, in 5 of 9 *N2*$^{F/LacZ}$*AlbCre* animals, large areas of bile infarcts were detected in the central parts of the liver (Fig. 5F). Furthermore, we detected considerable fibrosis in these mice as assessed by Sirius red staining. (Fig. 5G,H).

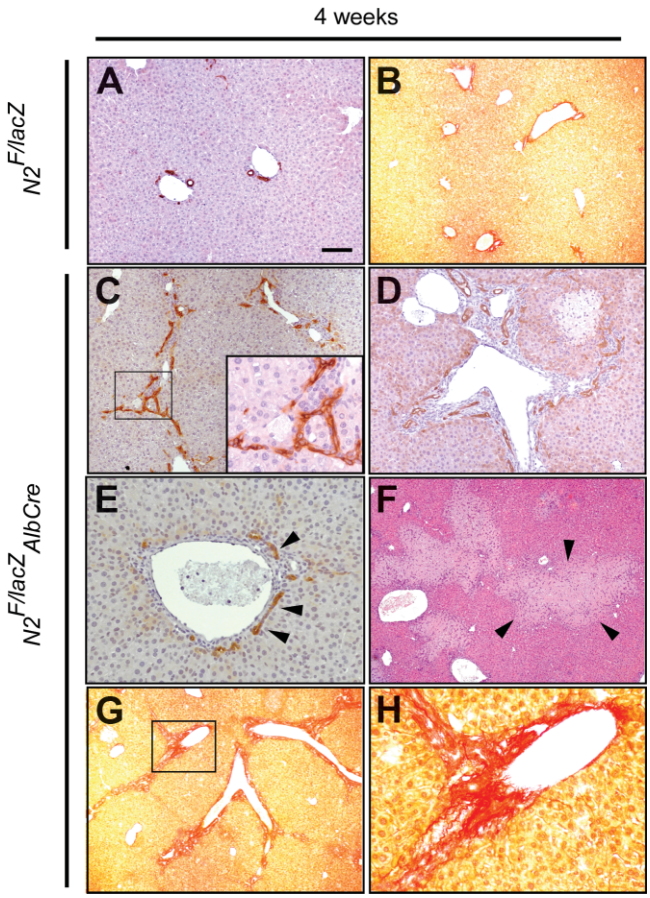

Fig. 5. Severity of the phenotype observed in *N2*$^{F/F}$*AlbCre* mice is enhanced in *Notch2* conditional/mutant (*N2*$^{F/LacZ}$*AlbCre*) animals. (A,B) Liver architecture and IHBD status in control heterozygous *Notch2*$^{F/LacZ}$ mutants are phenotypical WT as assessed with pan-CK immunostaining for detection of bile ducts and Sirius red staining to detect collagen content. (C) Four-week-old *Notch2* conditional/mutant (*N2*$^{F/LacZ}$*AlbCre*) animals show a similar IHBD status as observed in conditional *Notch2* (*N2*$^{F/F}$*AlbCre*) mice with the detection of multiple irregular ductules in pan-CK immunohistochemistry. The outlined area is magnified in the inset. (D-E) Large central portal tracts were also typically severely affected by IHBD malformations. (D) Pan-CK immunostaining reveals a large portal tract surrounded by severe inflammation and irregular bile ducts. (E) Frequently, large portal tracts did not contain any tubular biliary epithelial cells only displaying nonremodeled primitive ductal plate structures (arrowheads) as shown via pan-CK immunostaining. (F) Hematoxylin-eosin staining revealed large areas of central and peripheral bile infarcts (arrowheads) in *N2*$^{F/LacZ}$*AlbCre* animals. (G) Low-magnification view shows intense collagen deposits in portal and periportal areas of *N2*$^{F/LacZ}$*AlbCre* animals. The outlined area in panel G is magnified in panel H. Scale bar in panel A = (H) 25, (E) 50, (A,C,D) 100, and (B,F,G) 200 μm.

Discussion

In this study, we analyzed the role of liver-specific *Notch1* and *Notch2* ablation to hepatobiliary development and homeostasis. Encouraged by recent data that Notch1 might function as a tumor suppressor in hepatocytes,[12,31] we originally intended to study the role of Notch1 and Notch2 signaling in hepatocyte homeostasis using *AlbCre* mice. Because albumin expression is restricted to hepatocytes in the adult liver, and because the *AlbCre* mouse is widely used to "hepatocyte-specifically" induce Cre expression, we were surprised to detect normal hepatocytes but severe morphogenesis defects of the bile system in double-mutant *N1N2*$^{F/F}$*AlbCre* animals. How-

ever, because both hepatocytes and bile ducts originate from hepatoblasts, we found Cre-recombinase activity in hepatocytes and bile ducts but not in mesenchymal, endothelial, or hematopoetic cells when crossing *AlbCre* mice with the *Rosa26* reporter mouse. Thus, in agreement with previous reports,[27,28] the transgenic *AlbCre* mouse line is suitable for targeted disruption of *floxed* alleles in both hepatocytes and biliary epithelial cells.

In all our mouse strains lacking liver *Notch2* we detected a strong reduction of mature, regularly shaped bile ducts but observed abundant disorganized pan-CK–positive ductular structures along with impaired early postnatal remodeling and persistence of ductal plate structures. Biliary and structural abnormalities found were frequently accompanied by local cholestasis, feathery necrosis, portal inflammation, and enlarged portal tract expansion with collagen deposits. Because these findings were identical in $N1N2^{F/F}$*AlbCre* and $N2^{F/F}$*AlbCre* animals and further aggravated in $N2^{F/LacZ}$*AlbCre* mice, but were absent in $N1^{F/F}$*AlbCre* or $N1^{F/F}N2^{F/+}$*AlbCre* mice, we conclude that impaired Notch2 but not Notch1 signaling is responsible for the observed liver pathology. The pan-CK–positive duct-like structures were sometimes reminiscent of a ductular reaction, especially in the periphery of the portal area. Just like reactive ductular cells found in various states of the diseased liver,[32] these irregular structures found in mice lacking liver *Notch2* could possibly have arisen from preexisting cholangiocytes or their precursors, but also from progenitor cells such as oval cells. We detected Cre-induced recombination events in these abnormal ductular structures when analyzing $N1N2^{F/F}$*Rosa26AlbCre* mice, suggesting that they originate from albumin-expressing precursors, just like normal cholangiocytes. Together with the observation of ductal plate anomalies in *Notch2*-deficient mice, it is tempting to reason that these irregular pan-CK–positive ductules also originate from *Notch2*-deficient biliary epithelial cells or their precursors and are the result of impaired *Notch2*-dependent bile duct maturation and morphogenesis. However, we cannot fully rule out that an oval cell response contributes to the irregular duct-like structures observed, because albumin expression has been observed in rodent oval cells as well.[33,34]

Liver and bile duct development or maintenance of tissue integrity in the adult liver appeared perfectly normal in $Notch1^{F/F}$*AlbCre* mice. Interestingly, we did not find spontaneous hepatocyte proliferation or enlarged liver mass in the mouse strains lacking *Notch1*, specifically in the liver. This is somewhat surprising with respect to findings in mice with postnatal inactivation of *Notch1* ($N1^{F/F}$*MxCre* animals) using the interferon-α (IFN-α)–inducible *MxCre* promoter.[12] In that study, deletion of *Notch1* caused a striking eight-fold increase in hepatocellular proliferation accompanied by a 40% increase in liver mass.[12] One explanation for differences in hepatocyte proliferation as compared to transgenic *AlbCre* mice might be that IFN-α–induced activation of the *MxCre* promoter is not hepatocyte-specific. Rather, recombination of floxed alleles occurs in all tissues after IFN-α injection or likewise in all IFN-α–responsive tissues after polyl-ic injection most effectively in lymphatic tissues and the liver, including hepatocytes and nonparenchymal cells.[23,35] Because classic Notch signaling has been shown to inhibit hepatocyte growth factor (HGF) expression in vitro,[36] deletion of *Notch1* in cell compartments other than hepatocytes such as liver mesenchymal cells might alter expression of HGF within these cells and contribute to the enhanced proliferation of hepatocytes observed in $N1^{F/F}$*MxCre* animals.

In the conditional mouse strains investigated lacking a functional *Notch2* gene ($N1N2^{F/F}$*AlbCre* or $N2^{F/F}$*AlbCre* animals), the structural IHBD abnormalities were most pronounced in the periphery of the liver lobes, whereas in the central regions most portal tracts contained mature albeit frequently distorted bile ducts next to primitive pan-CK–positive ductular structures as well. Similar spatial disparities of IHBD morphology have also been described for human AGS, supporting the concept that Notch signaling is especially crucial for normal postnatal branching and elongation of IHBD.[37] However, it must be considered that in *AlbCre* animals, Cre-mediated deletion of *floxed* alleles occurs progressively with age[30] and bile duct development and morphogenesis around larger central portal veins starts at around embryonic day 15 before development of the finer branches of the biliary tree. Thus, it may well be the case that embryonic Notch2 levels using *AlbCre* mice still allow largely regular development and morphogenesis of functional IHBD in the central parts of the liver, thus preventing mice from severe generalized cholestasis and liver damage. Hence, progressive and cumulative *AlbCre*-driven recombination of *floxed* alleles may lead to Notch2 levels below a threshold that allows normal differentiation and morphogenesis of IHBD only later during bile duct development of the finer branches, thus leading to cholestasis-associated morphological changes, predominantly in the liver periphery. In this context, after further reduction of embryonic *Notch2* gene dosage in hepatoblasts in $N2^{F/LacZ}$*AlbCre* mice, we also found the central parts of the liver severely affected by structural bile duct malformation in the majority of mice, including bile duct paucity accompanied by profound portal inflammation and large areas of bile infarcts. However, though we did not find any structural abnormalities in livers of heterozygous $N2^{+/LacZ}$ mice,

we cannot rule out that loss of one functional *Notch2* allele in cells other than hepatoblasts and biliary precursors may contribute to the more severe phenotype observed in $N2^{F/LacZ}AlbCre$ animals.

Although *Notch2*-deficient livers displayed a strong reduction of normally formed, well-maturated bile ducts, we observed an increase of disorganized primitive biliary-like structures together with portal inflammation, portal tract enlargement and fibrosis, and biliary necrosis. These morphological changes are typical for chronic cholestasis but are less common in AGS, which is characterized by actual bile duct paucity without a marked inflammatory response and development of fibrosis in the majority of cases, in contrast to other cholangiopathies such as biliary atresia.[3,6,38] Actual ductopenia has also been described in mice doubly heterozygous for *Jagged1* and *Notch2* mutations (*Jagged1Notch2*$^{+/-}$ mice) as assessed by DBA staining.[14] *Jagged1Notch2*$^{+/-}$ animals also displayed severe heart defects and only 50% survived beyond P7. Those animals reaching adulthood, though displaying ductopenia, showed only modest portal tract enlargement with an increased number of periportal epithelial cells that had not been further characterized but possibly resemble those pan-CK–positive biliary-like cells we observed in *Notch2*-deficient livers. Loomes et al.[15] also described a strongly increased number of disorganized biliary epithelial cells together with marked portal tract enlargement in 50% of $Jagged1^{F/-}AlfpCre$ mice. Morphologically, these lesions equate those observed in our mouse model lacking liver *Notch2*. However, penetrance was lower, and expressivity of bile duct abnormalities and associated liver pathology seems less pronounced in $Jagged1^{F/-}AlfpCre$ mice compared with our mouse strains lacking liver *Notch2*, presumably due to residual Jagged1 expression of liver endothelial cells.[15] It remains unclear whether the different phenotype in our mouse model compared with human AGS or *Jagged1Notch2*$^{+/-}$ animals are attributable to cell-specific disruption of Notch2 signaling in our model, while Notch signaling is affected in all cell types, including cells of the hepatic reparative complex[3,38] in *Jagged1Notch2*$^{+/-}$ animals and in AGS patients. Impaired Jagged1 signaling via Notch receptors other than Notch1 and Notch2 might also contribute to the different liver pathology in *Jagged1Notch2*$^{+/-}$ animals or in AGS patients. Nevertheless, the sporadic finding of *Notch2* mutations in *Jagged1* mutation negative AGS patients,[39] together with our findings that cell-specific disruption of *Notch2* in livers of mice with WT genetic *Jagged1* background leads to developmental IHBD abnormalities, underscore a central role for *Notch2* in bile duct development.

How does impaired Notch2 signaling in biliary precursor cells lead to impaired IHBD development? Two sequential steps are necessary for IHBD formation: lineage commitment of hepatoblasts to differentiate to biliary epithelial cells, and further morphogenesis and maturation to form the intrahepatic biliary tree. The detection of ductal plate cells and biliary epithelial structures in all conditional *Notch2*-deficient mouse strains—including *Notch2* conditional/mutant $N2^{F/LacZ}AlbCre$ animals—suggests that Notch2 is not decisive for initial lineage commitment of hepatoblasts to biliary epithelial cells, although we cannot rule out that residual Notch2 even in $N2^{F/LacZ}AlbCre$ animals might suffice for this process during embryogenesis. Nevertheless, Notch2 signaling seems especially important for normal ductal plate remodeling and further maturation of primitive biliary structures to mature bile ducts. We speculate that Jagged1 signals from adjacent portal vein and hepatic artery endothelial cells[13,14] are necessary to properly guide bile duct development along portal veins, thus leading to disorganized biliary structures once Notch2 signaling is impaired in biliary epithelial cells. *In vitro* data obtained from cultivated hepatoblasts showed that Notch signals down-regulate CCAAT-enhancer-binding protein-α expression in cultivated hepatoblasts,[40] providing a possible molecular link to the impaired IHBD development in *Notch2*-disrupted livers, because CCAAT-enhancer-binding protein-α has been suggested to negatively regulate expression of hepatocyte nuclear factor 1β[41] and hepatocyte nuclear factor 6,[42] both of which are essential for normal IHBD morphogenesis.[41,43]

In conclusion, we provide evidence that single targeted disruption of *Notch2*, but not *Notch1*, leads to impaired IHBD development, supporting a central role of Notch2 in biliary cell maturation and morphogenesis. Additional genetic and *in vitro* studies are required to further unravel the molecular mechanisms to define the role of Notch1 and Notch2 in hepatobiliary development and disease.

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