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The Deletion of the Phosphatase Regulator NIPP1 Causes Progenitor cell Expansion in the Adult Liver

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Financial Support: This work was supported by the Belgian Foundation Against Cancer, FWO grant G048212N and the Prime Minister's office [IAP7/13].; Abbreviations: BEC, Biliary Epithelial Cells; BrdU, 5'-bromo 2'-deoxyuridine; DDC, 3,5-diethycarbonyl-1,4-dihydroxycollidine; LPC, liver progenitor cell; NIPP1, nuclear inhibitor of PP1; PP1, protein phosphatase-1.
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Key words. bile-duct hyperplasia • ductular reaction • liver progenitor cells • NIPP1 • protein phosphatase 1

ABSTRACT

The *Ppp1r8* gene encodes NIPP1, a nuclear interactor of protein phosphatase PP1. The deletion of NIPP1 is embryonic lethal at the gastrulation stage, which has hampered its functional characterization in adult tissues. Here, we describe the effects of a conditional deletion of NIPP1 in mouse liver epithelial cells. *Ppp1r8*$^{-/-}$ livers developed a ductular reaction, *i.e.* bile-duct hyperplasia with associated fibrosis. The increased proliferation of biliary epithelial cells was at least partially due to an expansion of the progenitor cell compartment that was independent of liver injury. Gene-expression analysis confirmed an upregulation of progenitor cell markers in the liver knockout livers but showed no effect on the expression of liver-injury associated regulators of cholangiocyte differentiation markers. Consistent with an inhibitory effect of NIPP1 on progenitor cell proliferation, *Ppp1r8*$^{-/-}$ livers displayed an increased sensitivity to diet-supplemented 3,5-diethoxycarbonyl-1,4-dihydrocollidine, which also causes bile-duct hyperplasia through progenitor cell expansion. In contrast, the liver knockouts responded normally to injuries (partial hepatectomy, single CCl_4 administration) that are restored through proliferation of differentiated parenchymal cells. Our data indicate that NIPP1 does not regulate the proliferation of hepatocytes but is a suppressor of biliary epithelial cell proliferation, including progenitor cells, in the adult liver. STEM CELLS *2016; 00:000–000*

SIGNIFICANCE STATEMENT

The liver has an exceptional capacity to compensate for lost cells. Depending on the nature and duration of the underlying damage this is explained by the ability of differentiated parenchymal cells or their progenitor cells to proliferate and differentiate. Here we show that the genetic deletion of the phosphatase regulator NIPP1 in liver epithelial cells causes a hyperproliferaton of periportal progenitor cells in the adult mouse liver. The loss of NIPP1 also sensitizes the liver to chemically induced progenitor cell proliferation. Our data identify NIPP1 as a novel suppressor of stem-cell proliferation in the postnatal liver.

INTRODUCTION

The liver shows an unusual capacity to recover from injury [1]. This is partially explained by the ability of parenchymal cells to re-enter the cell cycle. An alternative pathway for cell replenishment involves the proliferation of liver progenitor cells (LPCs) that can differentiate into hepatocytes or cholangiocytes. The relative contribution of LPCs and parenchymal cells to the replacement of lost cells depends on the type and duration of the inflicted damage [2]. Liver cell proliferation and differentiation is regulated by protein phosphorylation signaling cascades [3]. Several of the involved protein kinases have already been characterized but the counteracting phosphatases have not been studied yet. Protein phosphatase PP1 catalyzes more than half of all protein Ser/Thr dephosphorylation events in eukaryotic cells [4]. It forms multimeric complexes with dozens of regulatory proteins that determine when and where the phosphatase acts. One of the major nuclear interactors of PP1 is NIPP1, which is encoded by *Ppp1r8* and is expressed in (nearly) all eukaryotic cells. NIPP1 recruits a small subset of phosphoproteins, including transcription and pre-mRNA splicing factors, for regulated dephosphorylation by associated PP1 [5–7]. The deletion of NIPP1 in mice is early embryonic lethal [8], which has prevented its functional characterization. Here, we describe the phenotype of the targeted deletion of NIPP1 in liver epithelial cells. Liver development and regeneration from parenchymal cells were not affected by the deletion of NIPP1. Surprisingly, adult NIPP1$^{-/-}$ livers gradually developed bile-duct hyperplasia, associated with an expansion of the LPC compartment. Our data identify NIPP1 as a novel regulator of LPC proliferation.

MATERIALS AND METHODS

Provided as Supporting Information.

RESULTS AND DISCUSSION

Generation of liver-specific *Ppp1r8* knockout mice

The inactivation of one allele of *Ppp1r8* does not affect the NIPP1 transcript and protein levels, and causes no overt phenotype [8]. This enabled us to adopt a strategy for the conditional inactivation of *Ppp1r8* through excision of a single floxed allele, without the risk for phenotypic artifacts induced by the *Cre* transgene in the knockouts [9, 10]. First, *Ppp1r8*$^{+/-}$ mice were crossed with transgenics expressing *Cre recombinase* under control of the albumin promoter, with enhancer elements from both the albumin and α-fetoprotein genes (*Alfp-Cre*) [9, 11]. Subsequently, *Alfp-Cre*$^{+/-}$*/Ppp1r8*$^{+/-}$ animals were crossed with mice that had been engineered to contain *Cre*-targeted loxP sites (fl) flanking the promoter region and exon 2 of both *Ppp1r8* alleles (*Ppp1r8*$^{fl/fl}$). *Alfp-Cre*$^{+/-}$*/Ppp1r8*$^{fl/+}$ and *Alfp-Cre*$^{+/-}$*/Ppp1r8*$^{fl/-}$ mice were used as controls (CTRs) and liver-specific knockouts (LKOs), respectively (Figs. 1A, B; Supporting Information Fig. 1). Albumin (*Alb*) and α-fetoprotein (*Afp*) are expressed in hepatoblasts starting at E10.5 [11]. Hence, *Cre*-recombination results in the loss of NIPP1 from hepatoblast-derived epithelial cells, *i.e.* hepatocytes, cholangiocytes and their bipotential liver progenitor cells, which together constitute ≈ 90% of the liver mass. Both immunoblotting (Fig. 1C) and immunostaining (Figs. 1D, E) confirmed the disappearance of NIPP1 from liver nuclei in the LKOs. The level of NIPP1 was already decreased by some 50% at E14.5 and further reduced to ≈10% in mice of 2 and 12 months. This remnant level can be explained by the expression of NIPP1 in non-epithelial cells. In addition, some parenchymal cells in the LKOs still expressed NIPP1, hinting at a rare escape mechanism from *Cre*-recombination and/or infiltration of NIPP1 expressing cells from extrahepatic tissues.

Ppp1r8$^{-/-}$ livers develop bile-duct hyperplasia

Ppp1r8 LKO mice were born according to the expected Mendelian ratio. Hematoxylin-Eosin (H&E) stainings on paraffin-embedded liver sections showed no differences between CTR and LKO mice of 14 days (Supporting Information Fig. 2). However, by the age of two months the LKOs displayed an obvious form of bile-duct hyperplasia (Fig. 2A). In mice of 12 months the *Ppp1r8*$^{-/-}$ livers showed supernumerary, often dilated bile ducts that were still largely confined to the portal areas. The larger bile ducts had clearly recognisable lumina and did not appear to be obstructed. The ductular reaction in the LKOs was associated with biliary fibrosis at 2 and 12 months, as illustrated by collagen staining with Sirius Red (Figs. 2B, C). Bile-duct hyperplasia in the LKOs was independently confirmed by both immunostainings (Figs. 2D, E) and immunoblotting (Figs. 2F, G) for KRT19 (Cytokeratin-19). Indeed, the KRT19 immunostainings revealed an increased number of bile-duct epithelial cells (BECs), including periportal patches that represent LPCs, as revealed by the KRT19 positive area (Fig. 2E) and the pathological score (Supplementary Information Fig. 3). Immunoblot analysis showed a global increase of KRT19 in total liver lysates after ablation of NIPP1 (Figs. 2F, G). A severalfold LKO-associated overexpression of the *Krt19* transcript was also detected by qRT-PCR but no effect on the hepatocyte-specific marker albumin was found (Fig. 2H). Stainings for KI67 confirmed the increased BEC proliferation index in LKOs (Fig. 2I). Since ductular reactions are often associated with liver injury [1], we also examined the blood plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). These biochemical indicators of liver injury did not reveal significant differences between the CTR and LKO groups (Supplementary Information Fig. 4). It thus appears that the ductular reaction in adult *Ppp1r8*$^{-/-}$ livers occurs in

the absence of evident hepatocellular injury. Furthermore, macro- and microscopic histological analysis of LKO liver sections showed that bile-duct hyperplasia did not result in intrahepatic cholangiocarcinoma tumor development over time. Finally, we have explored whether immune cells, as key players in liver fibrosis, were altered upon the deletion of NIPP1 [12]. Immunostaining for markers of leukocytes (CD45) and activated hepatic stellate cells (αSMA) showed no difference; while immunostaining for the F4/80 receptor revealed that the macrophage population was enriched in the livers of the LKOs at 12 months (Supplementary information Fig. 5). The two major groups of the hepatic macrophages are the resident hepatic Kupffer cells and the infiltrating monocyte-derived macrophages. The latter population, which is increased following hepatic injury, is linked to both progression and resolution of fibrosis through promoting and abrogating the deposition of extracellular matrix, respectively [12, 13]. Taken together, these data suggest a contribution of the macrophage population to fibrosis.

The ductular reaction in *Ppp1r8*$^{-/-}$ livers is associated with LPC proliferation

To get an unbiased insight into the molecular mechanisms that underly the ductular reaction, we performed a comparative mRNA-microarray profiling of CTR and LKO liver samples at 2 months. SAM analysis revealed 417 upregulated and 43 downregulated genes in the LKOs (Fig. 3A, Supporting Information Table 1). Among the significantly upregulated genes in the LKOs were *Krt19* and some collagen isoforms, in accordance with the observed bile-duct hyperplasia and fibrosis (Fig. 3B, Supporting Information Table 1). Interestingly, *Tnfsf12*, which encodes the LPC proliferation inducer TWEAK, was significantly upregulated. TWEAK specifically stimulates LPC proliferation without hepatic damage [14]. In accordance with LPC proliferation, several established LPC marker genes were upregulated, including *Cd24a*, *Krt23* and *Prom1* [14–16]. In contrast, the transcripts of liver-injury associated regulators of cholangiocyte differentiation [17, 18], including *Il6*, *Tgfβ*, Notch target genes (*Hes1*, *Hey1/2*), *Hnf1β* and *Hnf6* were not affected by the loss of NIPP1. The microarray data were largely confirmed by qRT-PCR analysis (Fig. 3C). To examine whether the enhanced LPC proliferation requires signalling from other liver cell types, we examined the proliferation rate of the bipotential embryonic liver (BMEL) cells in culture. We found that siRNA-mediated knockdown of NIPP1, as verified by qRT-PCR (Fig. 3D), significantly increased the proliferation of BMEL cells *in vitro* in MTT assays (Fig. 3E). Collectively, these data suggest that the bile-duct hyperplasia in the LKOs can, at least partially, be explained by an expansion of the LPC compartment.

Ppp1r8$^{-/-}$ livers are sensitized to chemically induced LPC proliferation

Diet-supplemented 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) causes cholangiocytic injury and induces a ductular response through LPC proliferation [19]. The response to DDC feeding for 3 weeks was significantly increased in the *Ppp1r8*$^{-/-}$ livers, as deduced from H&E stainings (Fig. 4A), the KRT19 positive area (Figs. 4B, D) and the incorporation of BrdU (Figs. 4C, E). This sensitization to DDC is consistent with the notion of an enhanced LPC proliferation potential in *Ppp1r8*$^{-/-}$ livers. Carbon tetrachloride (CCl_4) is mainly toxic for hepatocytes [20]. CCl_4-induced liver damage for 24-48h caused a similar response in CTR and LKO mice with respect to increases in necrosis and BrdU-incorporation, and plasma markers for liver damage and inflammation (Supporting Information Figs. 6A-D). Likewise, liver regeneration following a 60% hepatectomy, which is largely accounted for by division of remaining parenchymal cells [21], appeared normal in the LKOs, as deduced after 48h from quantification of the proliferation markers PCNA and Ser10-phosphorylated histone H3 (Supporting Information Fig. 7). Thus, the loss of NIPP1 did not measurably affect the proliferation of liver parenchymal cells.

CONCLUSIONS

The deletion of NIPP1 in liver epithelial cells resulted in the development of bile-duct hyperplasia. BECs are heterogeneous with respect to their extent of differentiation and even differentiated cells can still divide [22]. However, our data strongly indicate that the increased proliferation of BECs in the LKOs is largely due to expansion of the LPC compartment and not linked to liver damage. Thus, NIPP1 probably plays a role in the maintenance of the stem-cell compartment rather than in the liver regeneration process *per se*. The ductular response in *Ppp1r8*$^{-/-}$ livers was associated with fibrosis, which results from an increased deposition of extracellular matrix components by hepatic stellate cells [1, 22]. Since the deletion of NIPP1 was selective for epithelial cells, it seems likely that fibrosis was a secondary response due to altered signaling from parenchymal to mesenchymal cells. Strikingly, the deletion of NIPP1 hampered the proliferation of embryonic stem cells [8] but promoted the proliferation of LPCs and BMEL cells (this work). This suggests that PP1-NIPP1 dephosphorylates distinct subsets of substrates in embryonic and adult stem cells. Future studies are aimed at identifying these substrates and elucidating their role in stem-cell proliferation.

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AUTHOR CONTRIBUTIONS

S.B.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; I.V.: collection and/or assembly of data, data analysis and interpretation; S.V.: collection and/or assembly of data, data analysis and interpretation; K.S.: collection and/or assembly of data, data analysis and interpretation; M.F.: collection and/or assembly of data, data analysis and interpretation; T.G.: collection and/or assembly of data; M.B.: conception and design, collection and/or assembly of data; T.R.: data analysis and interpretation; L.A.v.G.: conception and design, data analysis and interpretation; A.V.E.: conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript; M.B.: conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript, project coordination

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Figure 1. The inactivation of *Ppp1r8* in liver epithelial cells. (A) *Ppp1r8* alleles in CTR and LKO mice. **(B)** Efficiency and specificity of Cre recombination (Rec), as shown by PCR on tail and liver DNA from mice of 2 months, using primers 3, 4 and 6 (Supporting Information Fig. 1B). **(C)** NIPP1 immunoblotting in liver extracts from CTR and LKO mice. TBP served as a loading control. **(D, E)** NIPP1 (green) and DAPI (red) stainings of liver sections from CTR and LKO mice with the indicated ages. Arrowheads, NIPP1-positive cells. v, vein; b, bile duct. Scale bar, 100 µm.

Figure 1

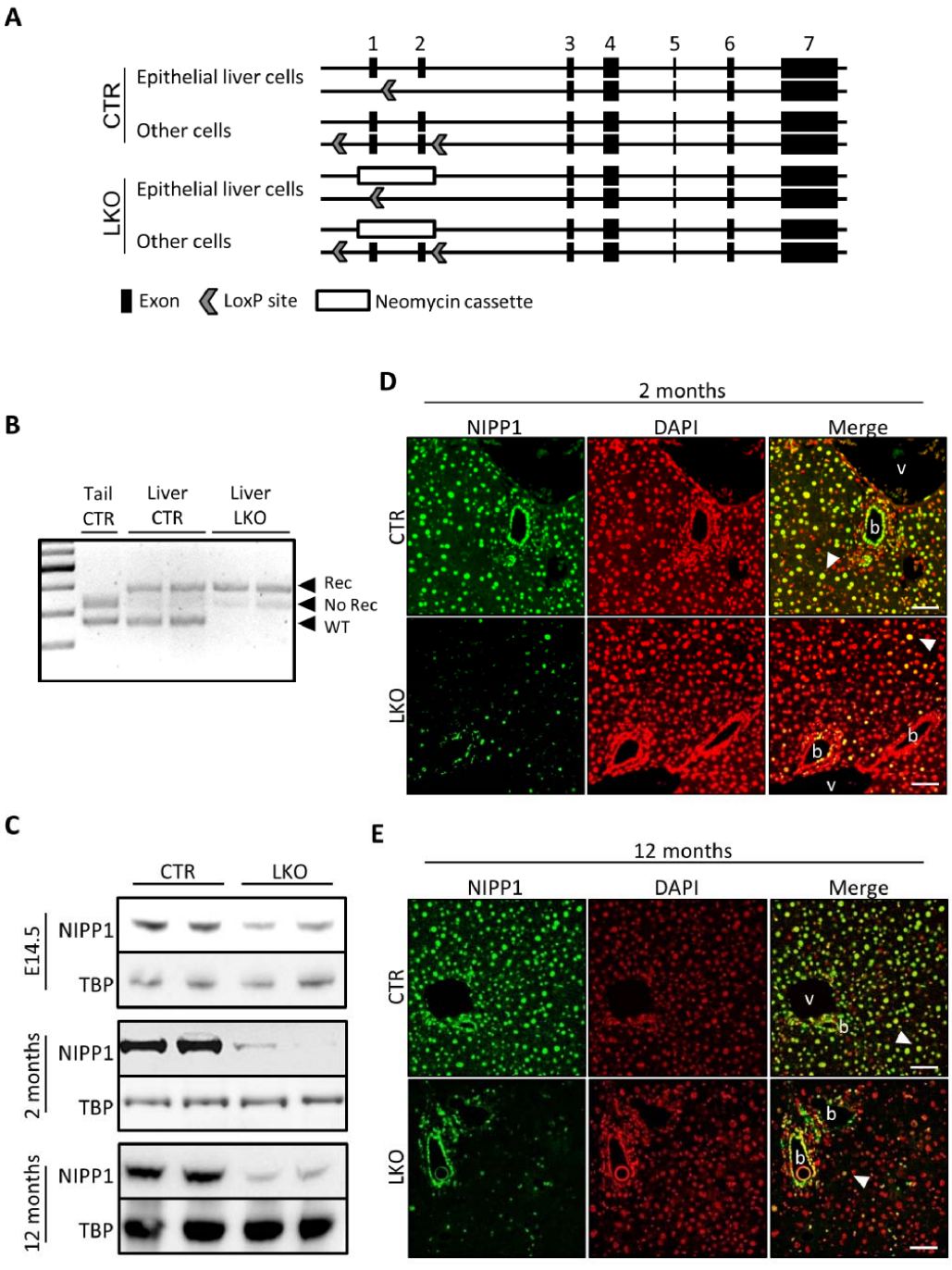


Figure 2. *Ppp1r8*$^{-/-}$ livers develop a ductular reaction. Liver sections of CTR and LKO mice of 2 and 12 months were stained with H&E (A), Sirius red **(B)** or KRT19 (**D**). The insets in panel D show a higher magnification of KRT19-positive progenitor cells (*) and bile ducts (b). Scale bar, 100 µm. **(C)** and **(E)** show quantifications of panels B and D, resp. The level of KRT19 in total liver lysates from both 2 and 12 months old CTR and LKO mice were visualized by immunoblotting (**G**) and quantified (**F**). GAPDH served as a loading control. **(H)** Relative *Alb* and *Krt19* transcript levels. *Hprt* was used for normalization. **(I)** Quantification of KI67 positive hepatocytes (HEP) and BECs.

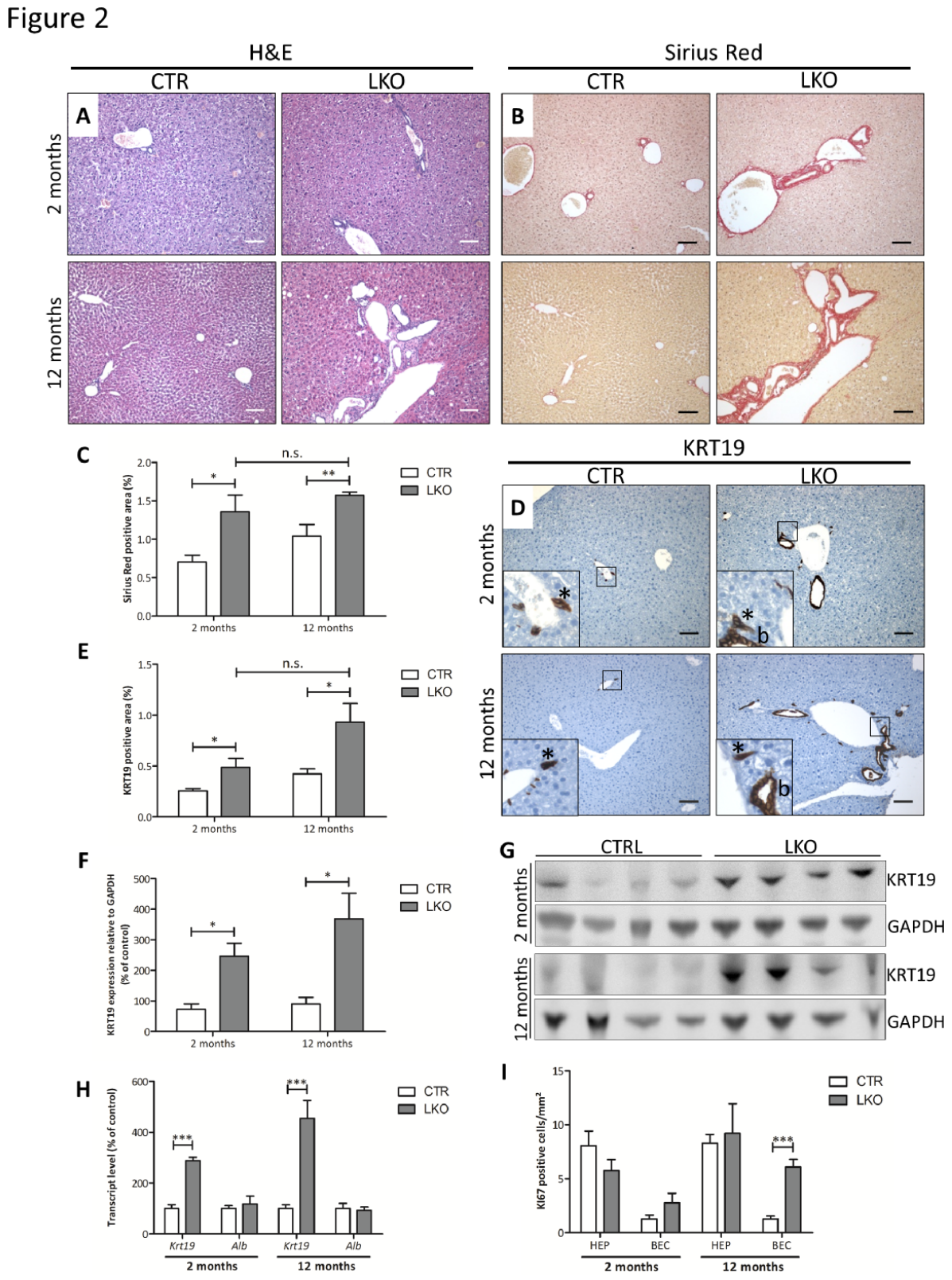


Figure 3. Upregulation of LPC marker genes in LKO mice. (A) Graphical representation of the up and downregulated genes in the LKOs. Microarray SAM analysis **(B)** or qRT-PCR **(C)** of the indicated LPC marker and regulators of cholangiocyte differentiation genes. Results represent means ± SE (n = 4). Knockdown of NIPP1 in BMEL (14B3) cells was performed by transfection with NIPP1-specific (KD) or non-targeted siRNA (CTR), and verified by qRT-PCR (**D**). The growth rate of these cells was determined by MTT assays (**E**) at the indicated time points and represented as means ± SE (n= 4). *, *P*<0.05; ***, *P*<0.001.

Figure 3

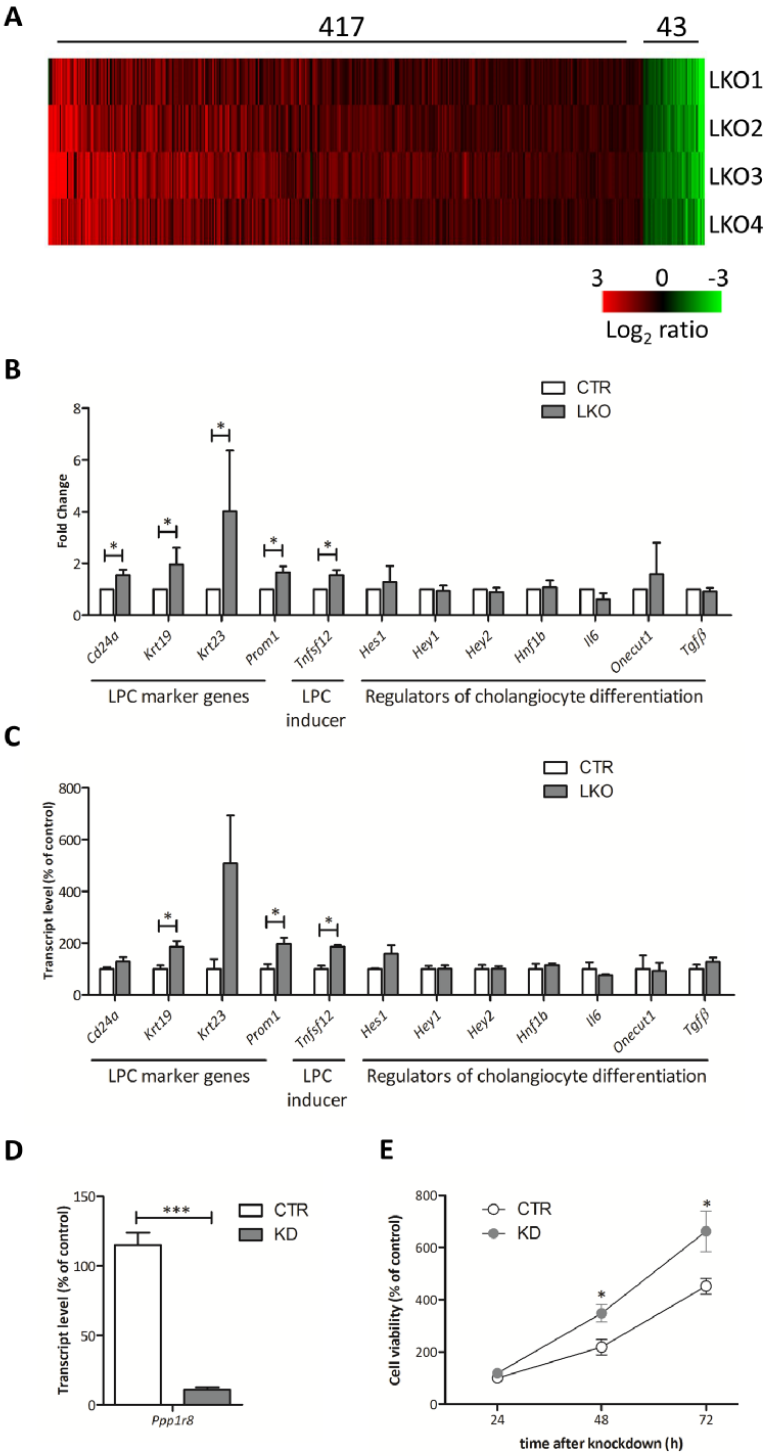
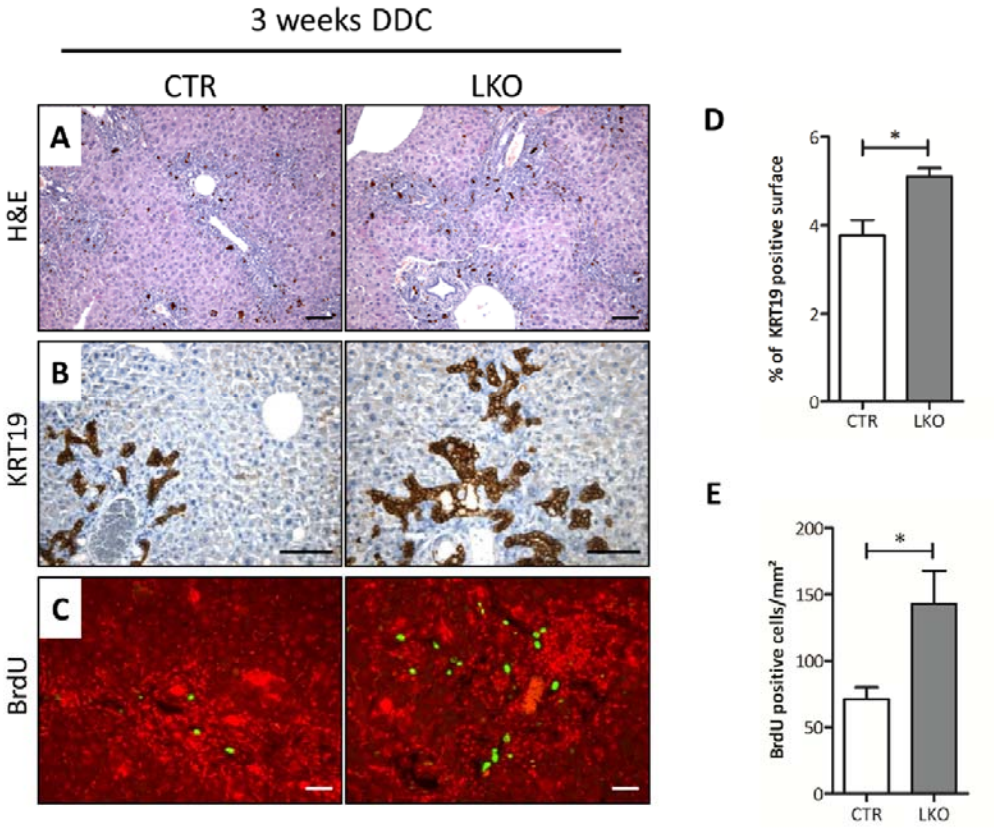
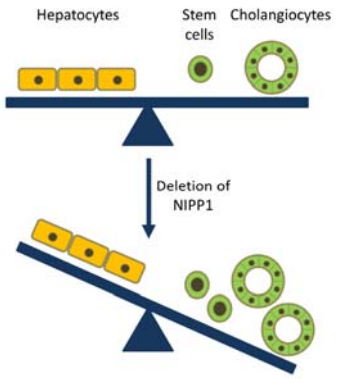


Figure 4. LKO mice show an enhanced bile-duct hyperplasia in response to DDC feeding. Liver sections of CTR and LKO mice fed for 3 weeks with a DDC diet were stained with H&E **(A)**, KRT19 **(B)** and for incorporated BrdU (green) in PI (propidium iodide) stained nuclei (red) **(C)**. Scale bar, 100 µm. **(D)** Quantification of the KRT19-positive surface in liver sections (n=4), as shown in panel B. **(E)** Quantification of BrdU positive cells (n=4), as shown in panel C. The bar data represent means ± SE. *, *P*<0.05.

Figure 4





Graphical Abstract

NIPP1 controls the balance between liver epithelial cells.