Article type : Original

Fine-tuning of SIRT1 expression is essential to protect the liver from cholestatic liver disease.

Short title: SIRT1 contributes to cholestasis

Britt A Blokker[1,2], Monica Maijo[2], Marta Echeandia[2], Mikel Galduroz[2], Angela M Patterson[2], Anna Ten[2], Mark Philo[3], Rebecca Schungel[2,4], Virginia Gutierrez-de Juan[5], Emina Halilbasic[6], Claudia Fuchs[6], Gwenaelle Le Gall[3], Malgorzata Milkiewicz[7], Piotr Milkiewicz[8], Jesus M Banales[9], Simon M Rushbrook[10], José M Mato[5], Michael Trauner[6], Michael Müller[1], María Luz Martínez-Chantar[5], Marta Varela-Rey[5] and Naiara Beraza[2,5,11]

[1]Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, UK. [2]Gut Microbes and Health Research Programme, Quadram Institute, Norwich Research Park, Norwich, UK. [3]Metabolomics Unit, Quadram Institute, Norwich Research Park, Norwich, UK. [4]Department of Food, Nutrition, Facilities, University of Applied Sciences Münster, Münster, Germany [5]CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Derio, Bizkaia, Spain. [6]Hans Popper Laboratory of Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University Vienna, Vienna, Austria. [7] Department of Medical Biology, Pomeranian Medical University, Szczecin, Poland. [8]Liver

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/hep.30275

and Internal Medicine Unit, Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Poland. [9]Department of Liver and Gastrointestinal Diseases, Biodonostia Health Research Institute, Donostia University Hospital, University of the Basque Country (UPV/EHU), CIBERehd, Ikerbasque, Donostia, Spain. [10]Department of Gastroenterology, Norfolk and Norwich University Hospital, Norwich, UK

[11]To whom correspondence should be addressed.

Corresponding Author:

Naiara Beraza, PhD

Gut Health & Food Safety Research Programme, Quadram Institute, Norwich Research Park, Norwich, NR4 7UA UK.

e-mail: naiara.beraza@quadram.ac.uk

Tel. 0044(0)1603251413

Keywords: Sirtuin1; cholestasis; FXR; bile acids; NorUDCA

Financial support:

This research was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) by the BBSRC Institute Strategic Programme Gut Health and Food Safety and Gut Microbes and Health BBS/E/F/00044509 (to NB), the Science Links Seed Fund grant (to NB, SR), Biotechnology and Biological Sciences Research Council (BBSRC) Doctoral Training

Partnership programme (to NB, AT), and Instituto de Salud Carlos III; FIS, PS12/00402 (to NB and MVR). NB was funded by the BBSRC Institute Strategic Programme Gut Health and Food Safety BB/J004529/1, the Program Ramon y Cajal (Ministry of Economy and Competitiveness, Spain) and Ikerbaske foundation (Basque government, Spain). FIS PI12/00380, FIS PI15/01132 and Miguel Servet Program CON14/00129 cofinanced by "Fondo Europeo de Desarrollo Regional" (FEDER) (to JMB). Gobierno Vasco-Departamento de Salud 2013111114 (to M.L.M.-C), MINECO: SAF2014-54658-R, EITB Maratoia BIO15/CA/014 (to M.L.M.C) Asociación Española contra el Cáncer. Ciberehd is funded by the Instituto de Salud Carlos III. Work produced with the support of a 2017 Leonardo Grant for Researchers and Cultural Creators, BBVA Foundation (to MVR).

ME and MG were supported by the Camara de Comercio de Navarra

Conflict of Interest Statement: All authors declare that they have no competing financial interests with respect to this manuscript.

Abstract

Cholestasis comprises aetiologically heterogeneous conditions characterized by accumulation of bile acids in the liver that actively contribute to liver damage. Sirtuin 1 (SIRT1) regulates liver regeneration and bile acid metabolism via modulating the farnesoid X receptor (FXR); we here investigate its role in cholestatic liver disease.

We determined SIRT1 expression in livers from patients with cholestatic disease, in two experimental models of cholestasis, as well as in human and murine liver cells in response to bile acid loading. SIRT1 overexpressing ($SIRT^{oe}$) and hepatocyte-specific SIRT1-KO mice ($SIRT^{hep-/-}$) were subjected to BDL and were fed with 0.1%DDC diet to determine the biological relevance of SIRT1 during cholestasis. The effect of NorUDCA was tested in BDL/$SIRT^{oe}$ mice.

We found that SIRT1 was highly expressed in livers from cholestatic patients, mice after BDL and Mdr2$^{-/-}$ animals. The detrimental effects of SIRT1 during cholestasis were validated *in vivo* and *in vitro.* SIRT^{oe} mice showed exacerbated parenchymal injury whereas SIRT$^{hep-/-}$ mice evidenced a moderate improvement after BDL and 0.1%DDC feeding. Likewise, hepatocytes isolated from SIRT^{oe} mice showed increased apoptosis in response to bile acids, while a significant reduction was observed in SIRT$^{hep-/-}$ hepatocytes. Importantly, the decrease, but not complete inhibition of SIRT1 exerted by NorUDCA treatment correlated with pronounced improvement in liver parenchyma in BDL/SIRT^{oe} mice. Interestingly, both SIRT1 overexpression and hepatocyte-specific SIRT1 depletion correlated with inhibition of the farnesoid X receptor (FXR), whereas modulation of SIRT1 by NorUDCA associated with restored FXR-signalling.

Conclusion: SIRT1 expression is increased during human and murine cholestasis. Fine-tuning expression of SIRT1 is essential to protect the liver from cholestatic-liver damage.

The term 'cholestatic liver disease' includes a broad spectrum of aetiologically heterogeneous hepatobiliary disorders, mainly comprising primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) in adults. These conditions are characterised by accumulation of bile acids in the liver, leading to hepatocellular necrosis and apoptosis, progressive fibrosis and end-stage liver disease(1-3). Current therapeutic approaches for treating cholestasis mainly rely on the use of Ursodeoxycholic acid (UDCA); however, this treatment has no proven efficacy for PSC and a proportion of patients with PBC(2, 3). The therapeutic options for such unresponsive patients are currently limited though there have been recent promising advances including the use of 24-norursodeoxycholic acid (NorUDCA)(4), which has shown to improve liver function in PSC patients in a recent clinical trial(5). Also, novel treatments using fibrates(6) and FXR agonists, such as obeticholic acid(7-9), have shown efficacy for PBC patients unresponsive to UDCA. Still, a better

understanding of the molecular mechanism underpinning the pathogenesis of cholestasis will enable the development of efficient therapies for cholestatic patients.

FXR is an orphan nuclear receptor that plays a key role in the regulation of bile acid metabolism, and in the pathogenesis of cholestasis (6, 10-13). The regulation of FXR involves a dynamic acetylation/deacetylation process mediated by p300 and Sirtuin 1 (SIRT1) respectively(14). SIRT1 deacetylates FXR, increasing its DNA binding and dependent gene transcription. Interestingly, SIRT1/FXR interaction must be finely tuned, as prolonged SIRT1-mediated FXR deacetylation leads to ubiquitination and proteasome degradation(14).

SIRT1 is an evolutionarily conserved NAD^+-dependent histone III deacetylase that is activated in response to energy deprivation, controlling key metabolic functions including bile acid metabolism(15, 16). Initial work delineating the implication of SIRT1 in prolonging the lifespan in lower organisms(17) and in promoting healthy ageing in mammals(18) led to SIRT1 being hyped as a 'magic bullet' to preserve lifelong health. Nevertheless, the role of SIRT1 has revealed to be highly complex in a wide range of biological functions including tumorigenesis. We and others have described SIRT1 as being highly expressed in human liver tumours(16, 19, 20), pointing to the potential contribution of SIRT1 to liver disease. Supporting this, we demonstrated that SIRT1 overexpression leads to impaired liver regeneration after partial hepatectomy, which associated with disturbances in bile acid homeostasis, including reduced FXR signalling, increased synthesis and accumulation of toxic bile acids in the liver(16). Overall, these results led us to hypothesise that SIRT1 may play a role during cholestatic liver disease.

In accordance, in this study, we provide evidence that SIRT1 is up-regulated in the liver during human cholestasis in PSC and PBC patients and in two murine models of cholestasis; after bile duct ligation (BDL) and in $Mdr2^{-/-}$ mice. We further demonstrate that SIRT1 contributes to liver parenchymal damage in the context of obstructive cholestasis, as

the overexpression of SIRT1 aggravates liver injury, whereas hepatocyte-specific SIRT1 depletion exerts a moderate cell protection after BDL and feeding with a diet containing 0.1% of DDC. Importantly, the improvement in liver function observed in hepatocyte-specific SIRT1 KO mice is only transient, likely involving mechanisms including the attenuation of FXR signalling. Ultimately, we describe that the beneficial effect of NorUDCA treatment in reducing liver injury in cholestatic SIRT1 overexpressing mice, associates with the modulation, whilst not complete depletion, of SIRT1 expression.

Overall, our results support the importance of maintaining SIRT1 fine-tuned expression to preserve liver function in the context of cholestatic disease.

MATERIALS AND METHODS

Human PBC and PSC samples

SIRT1 gene expression was determined by qPCR analysis in mRNA isolated from cirrhotic livers of patients with PBC (n=10) and PSC (n=10) who underwent liver transplantation. Control liver tissues (n=5) were acquired from large margin liver resections from patients undergoing of colorectal metastases with no microscopic changes of liver disease identified by a pathologist, all collected in the Department of General, Transplant and Liver Surgery, Medical University of Warsaw, Poland as described elsewhere(21). Supplemental Table 1 includes detailed clinical and biochemical data of these patients.

Protein expression of SIRT1 was assessed by immunohistochemistry (IHC) in paraffin embedded sections from livers obtained by percutaneous biopsy from n=9 PBC patients, n=5 PSC patients and in liver samples obtained from n=4 healthy individuals at the Norwich Norfolk University Hospital, UK. The diagnosis was established by pathological analysis of liver biopsies together with presence of anti-mitochondrial antibodies in the case of PBC. Clinical and biochemical data of these patients is included in Supplemental Table 2. The use

of human tissue samples was approved by the Faculty of Medicine and Health Sciences Research Ethics committee (University of East Anglia, UK). Collection and handling of human samples used in this study conformed to the Declaration of Helsinki and the Human Tissue Act (UK) and Good Clinical Practice Guidelines (UK).

More information in **Supplemental Material and Methods.**

RESULTS

SIRT1 is up-regulated during human and murine cholestasis

The expression of SIRT1 during PBC and PSC, the main human cholestatic liver disease aetiologies, has not been characterised to date. As shown in Fig 1A, SIRT1 was highly expressed in cholestatic livers from PBC and PSC patients at the gene transcript level. Immunohistochemical analysis evidenced increased positive SIRT1 immunostaining mainly localised in the nuclei of hepatocytes and bile duct cells in PBC and PSC patients (Fig 1B, C). In contrast, lower and more diffuse SIRT1 staining was detected in livers from healthy individuals (Fig 1B, C). These results suggest that increased SIRT1 nuclear expression relates to the cholestasis itself and not to the specific aetiology of the disease.

To determine whether bile acids have a direct effect on triggering SIRT1 upregulation during cholestasis, we exposed THLE-2 cells (liver epithelial cells of human origin) to different bile acids including primary and secondary species, and found a significant increase in SIRT1 expression (Fig 1D).

Further studies in murine models of cholestasis confirmed that SIRT1 is upregulated at different timepoints after BDL at gene (Fig 2A) and protein level (Fig 2B-D, Suppl Fig. 1A) in WT mice (Fig 2C, D). No changes in SIRT1 expression were observed in livers from sham operated mice (Suppl. Fig 1B, C).

In accordance with our results in mice after BDL, analysis of liver tissue samples from $Mdr2^{-/-}$ mice, a well-established mouse model resembling PSC(22), showed increased number of hepatocytes expressing SIRT1, as evidenced by IHC and further quantification of positive hepatocytes (Fig 2F), and higher protein expression in nuclear liver extracts as shown by immunoblotting analysis (Suppl Fig 1D, E).

I*n vitro* studies in primary hepatocytes from WT mice supported our observations in human liver cells (Fig 1D), showing SIRT1 upregulation in response to CDCA DCA, GCA and CA at a dose of 125μM (Suppl Fig2A). Increased SIRT1 expression in hepatocytes associated with augmented apoptosis after bile acid load (Suppl Fig 2B), was not altered in the presence of caspase-3 inhibitor (Suppl Fig 2C), supporting that SIRT1 upregulation is not resulting from increased apoptosis. Further studies using the bile acid species with a higher impact on cell death showed that CDCA and DCA triggered AMPK phosphorylation (Suppl. Fig 2D). Inhibition of AMPK activity partially reduced SIRT1 expression (Suppl. Fig 2E) and decreased apoptosis (Suppl. Fig 2F). SIRT1 and AMPK are key metabolic regulators activated in response to changes in nutrient or energy availability(23, 24). Importantly, serum supplementation to the culture media reduced AMPK phosphorylation (Suppl. Fig 2G), SIRT1 expression (although still present) (Suppl. Fig 2H) and apoptosis (Suppl. Fig 2I) in response to bile acids.

Overall, our results indicate that SIRT1 expression increases during cholestasis, likely driven by the accumulation of bile acids, contributing to hepatocyte cell death.

SIRT1 overexpression aggravates liver injury, inflammation and fibrogenesis after bile duct ligation

To gain further insight into the biological relevance of increased SIRT1 expression during cholestatic liver disease, we performed BDL in mice that overexpress SIRT1 (Suppl Fig 3A, B) (hereafter, $SIRT^{oe}$ mice).

The increase in serum markers of liver function and the profuse presence of necrotic areas observed in $SIRT^{oe}$ mice evidenced the detrimental impact of SIRT1 overexpression during cholestasis (Fig 3A, B). Analysis of caspase-3 activity (Suppl. Fig 3C) and TUNEL assay (Fig 3C and Suppl. Fig 3D) supported that higher apoptotic cell death occurs after BDL in $SIRT^{oe}$ mice compared to WT animals.

In vitro analyses in isolated hepatocytes from $SIRT^{oe}$ mice confirmed that overexpression of SIRT1 further sensitizes liver cells to bile acid-induced apoptotic cell death (Fig 3D, Suppl. Fig 3E).

Characterisation of the inflammatory response by FACS analysis of liver-isolated immune cells showed that $SIRT^{oe}$ mice had higher presence of macrophages at 7days after BDL compared to WT mice (Fig 3E). Analysis of cytokine (IL1β, IL6, IFNγ), activation factors (NOS2), chemokine (CCL2) and chemokine receptor (CCR1, CCR2 and CCR5) expression confirmed the increased proinflammatory milieu in $SIRT^{oe}$ mice compared to WT after BDL. TNFα expression was comparable in both genotypes (Fig 3F). TNFα, IL6 and CCL2 ELISA confirmed the gene expression results obtained (Suppl Fig 3F).

Finally, fibrogenesis was assessed in $SIRT^{oe}$ and WT mice after BDL. Sirius red staining (Fig 3G and Suppl Fig 3G left panels), αSMA determination by IHC on liver sections (Fig 3G and Suppl. Fig 3G right panels) followed by quantification (Fig 3G) and qPCR analysis of Collagen 1A1, αSMA and TGFβ gene expression (Suppl Fig 3H), supported the increased fibrogenesis in $SIRT^{oe}$ animals compared to WT mice after BDL.

Analysis of WT and SIRT^{oe} mice at 3d and 7d after sham-surgery showed no significant differences compared to control animals in the parameters described above including, serum liver damage markers (Suppl Fig 4A), liver parenchyma status (Suppl Fig 4B), hepatocyte apoptosis (Suppl Fig 4C), inflammation (Suppl Fig 4D, E) and fibrosis (Suppl Fig 4 F, G). Macrophage counts and Caspase3 were slightly increased in Sham WT and SIRT^{oe} mice respectively compared to control mice, though these parameters were still significantly different in the BDL mice, supporting the specificity of the biological response observed after BDL.

Overall, our results evidence that SIRT1 overexpression aggravates liver injury, hepatocellular death, inflammation and consequent fibrogenesis in the context of cholestasis.

SIRT1 overexpression alters FXR-mediated regulation of bile acid synthesis

During cholestasis, FXR mediates compensatory responses aiming at inhibiting endogenous bile acid production and regulating their transport, in a coordinated manner with other nuclear receptors(12).

In line with the described crosstalk regulation(12-14), we found that SIRT1 overexpression associates with decreased FXR protein expression during cholestasis. While WT mice showed a transient increase in FXR at 3 days after BDL that ameliorated after 7days, SIRT^{oe} mice had persistently lower FXR levels (Fig 4A). Lower SHP and higher Cyp7A1 expression found in SIRT^{oe} mice compared to WTs at 3d after BDL confirmed the impaired FXR-signalling in the context of SIRT1 overexpression at this timepoint (Fig 4B).

Further analysis of liver bile acid content showed an enlarged pool size in SIRT^{oe} mice after BDL compared to WT animals (Fig 4C) while no significant differences were detected in faecal excretion (Suppl Fig 5A). Analysis of bile acid transporters showed no

significant differences between WT and SIRT^{oe} mice in Oatp, Ntcp, Bsep or Mrp4 expression (Fig 4D) supporting that increased bile acid accumulation in SIRT^{oe} mice resulted from higher synthesis.

Sham surgery had no impact on modulating FXR-signaling or bile acid transporters expression compared to untreated control mice with the exception of Ntcp, which expression was reduced in sham mice compared to control animals but was still significantly different from WT/BDL and SIRT^{oe}/BDL mice (Suppl Fig 5B-D).

Overall, our results demonstrate that SIRT1 overexpression contributes to the accumulation of bile acids in the liver during cholestasis upon attenuation of FXR-mediated inhibition of bile acid synthesis.

SIRT1 overexpression attenuates cholangiocyte proliferation

Liver cholestasis is characterised by chronic bile duct injury with proliferation of cholangiocytes (ductular reaction) at the early stages and later ductopenia(1, 3).

Interestingly, CK19 immunostaining on liver sections showed a moderate increase of the ductular reaction in WT compared to SIRT^{oe} mice (Fig 5A, B).

Aiming to determine how SIRT1 may influence cholangiocyte function we performed *in vitro* analysis of normal mouse cholangiocytes (NMC) where we induced the overexpression of SIRT1 by transfecting with a plasmid DNA (SIRT1^{oe}). An empty vector was transfected as control (Control). We found that SIRT1^{oe}/NMC showed similar apoptotic response to bile acid stimulation when compared to control/NMC (Fig 5C). Further analysis of cell cycle regulation revealed that SIRT1^{oe} NMC had lower cyclin D1 and E expression when cultured in the presence of growth factors, suggesting that SIRT1 overexpression may attenuate cell proliferation (Fig 5D). Finally, FACS analysis showing a higher percentage of

SIRT1^{oe}/NMC arrested in the G1 phase compared to control cells (Fig 5E, F) confirmed that SIRT1 overexpression attenuates cholangiocyte proliferation.

SIRT1 overexpression contributes to increased liver injury and fibrogenesis after 0.1% DDC-induced cholestasis

The detrimental impact of SIRT1 overexpression during cholestasis was further confirmed in an additional experimental model where 0.1%DDC-fed SIRT^{oe} mice showed increased Alkaline phosphatase serum levels (Suppl. Fig 6A) and wider areas of liver necrosis after 1 week of treatment (Suppl. Fig 6B). Though not prominent, 0.1%DDC/SIRT^{oe} showed increased cell death (Suppl Fig 6C, D) compared to 0.1%DDC/WT animals. As observed after BDL, SIRT^{oe} mice showed milder ductular reaction as evidenced by CK19 immunostaining (Suppl Fig 6E) and higher fibrosis (Suppl Fig 6F) than WT animals after DDC. Western blot analysis showed strong reduction of FXR in both genotypes after DDC diet although expression was found to be lower in SIRT^{oe} mice compared to WT animals (Suppl Fig 6G).

Overall, our results in this alternative model of cholestasis support the detrimental impact that SIRT1 overexpression has on liver damage during cholestasis.

Hepatocyte-specific SIRT1 depletion leads to a moderate but transient attenuation of cholestatic-liver injury after BDL

Our results indicate that SIRT1 overexpression contributes to aggravation of liver damage during cholestasis, pointing to the modulation of SIRT1 as a therapeutic approach. Next, we aimed to investigate how hepatocyte-specific SIRT1 depletion may impact on liver injury during BDL-induced cholestasis.

We found that hepatocyte-specific SIRT1 KO mice ($SIRT^{hep-/-}$) with only residual SIRT1 expression in the liver (Suppl Fig 7A, B) showed a moderate improvement on liver damage markers and the liver parenchymal status (Fig 6A, B), whereas liver injury seemed to reach comparable levels as WT mice at later time points (7 days) after BDL (Fig 6A, B). Quantification of the apoptotic response by TUNEL assay (Fig 6C, Suppl Fig 7C) and Caspase-3 activity (Suppl Fig 7D) showed a reduction of apoptotic cell death in $SIRT^{hep-/-}$ mice compared to WT mice after BDL.

In vitro analysis confirmed that hepatocytes isolated from $SIRT^{hep-/-}$ mice had a significantly lower apoptotic response to DCA and CDCA (Fig 6D), whereas the overall cell survival was lower in KO cells when compared to WT cells (Suppl Fig 7E), indicating an increase in necrosis. Interestingly, hepatocytes isolated from $SIRT^{hep-/-}$mice showed reduced pAMPK levels (Suppl Fig 7F) after bile acid stimulation supporting the crosstalk between SIRT1 and AMPK in response to bile acids. In line with the reduction in apoptosis upon SIRT1 depletion, inhibition of AMPK blunted bile-acid induced apoptosis (Suppl 7G).

Liver inflammation was comparable in $SIRT^{hep-/-}$ mice and WT animals after BDL as no significant differences in liver macrophages (Fig 6E) or in the expression TNF, IFNγ, IL10, NOS2, CCL2, CCR2 and CCR5 (Fig 6F, Suppl Fig 8A). The mild increase in IL6, IL1β and CCR1 in $SIRT^{hep-/-}$ mice was no statistically significant. We found no significant differences in the ductular reaction (Suppl Fig 8B, C) or the degree of fibrosis (Fig 6G and Suppl Fig 8D, E) in $SIRT^{hep-/-}$ mice compared to WT animals after BDL.

Interestingly, $SIRT^{hep-/-}$ mice had lower FXR protein expression 3 and 7 days after BDL compared to WT littermates (Fig 7A). Reduced FXR correlated with lower SHP and higher CYP7A1 expression in $SIRT^{hep-/-}$ mice at 3 days after BDL that were further regulated 7 days after surgery similarly in both WT and KO mice (Fig 7B). Accordingly with the increased SIRT1, we found reduced FXR acetylation at 3 and 7 days after BDL in WT mice (Suppl Fig 9A). In accordance with our observations, FXR acetylation and total protein

expression was further reduced in SIRT$^{hep-/-}$ mice (Suppl Fig 9A). Consequently, an increased accumulation of bile acids in the liver of SIRT$^{hep-/-}$ mice was observed after BDL (Fig 7C), though this did not reach statistical significance. The expression of bile acid transporters was comparable between SIRT$^{hep-/-}$ and WT mice after BDL (Fig 7D), supporting that the increased BA synthesis is a consequence of attenuated FXR signalling in the absence of hepatocytic-SIRT1 and not to changes in transport. Though not statistically significant, Mrp4 expression was slightly higher in SIRT$^{hep-/-}$ mice compared to WT animals, which could reflect a slight increase in the alternative transport of bile acids, overall impacting on the total bile acid pool size.

Sham surgery had no impact on modulating liver injury (Suppl Fig 10A-C), inflammation (Suppl Fig 10D, E), ductular reaction (Suppl Fig 10F) and fibrosis (Suppl Fig 10G, H) in SIRT$^{hep-/-}$ mice.

Overall, our results suggest that SIRT1-hepatocyte depletion exerts a degree of protection against bile acid-induced apoptosis but not necrosis, explaining the transient benefits observed during cholestasis *in vivo* in SIRT$^{hep-/-}$ mice.

Hepatocyte-specific SIRT1 depletion leads to a moderate but transient attenuation of cholestatic-liver injury after 0.1%DDC diet feeding

Further analyses in 0.1% DDC treated SIRT$^{hep-/-}$ mice confirmed our results obtained in the BDL experimental model as, despite the reduction in levels of liver damage serum markers in SIRT$^{hep-/-}$ mice compared to WTs (Suppl Fig 11A) no significant differences in parenchyma structure were detected (Suppl Fig 11B). Similarly to what was found after BDL, though not prominent, 0.1% DDC/SIRT$^{hep-/-}$ mice had lower apoptosis (Suppl Fig 11C, D) compared to 0.1% DDC/WTs. Finally, ductular reaction and fibrosis were comparable in SIRT$^{hep-/-}$ and WT mice 1 week after 0.1% DDC feeding (Suppl Fig 11E, F). Western blot

analysis showed decrease in FXR expression in both genotypes after DDC diet that was more pronounced in SIRT$^{hep-/-}$ mice (Suppl Fig 11G).

The beneficial effects of NorUDCA on attenuating cholestatic-liver injury associate with the reduction, but not inhibition, of SIRT1 expression

NorUDCA has proven efficacy in treating murine cholestasis in Mdr2$^{-/-}$ mice (25, 26) and improving cholestasis in PSC patients(5). We previously described that NorUDCA reduced SIRT1 expression in non-cholestatic SIRT^{oe} mice, which associated with an improved response to injury and restored regenerative capacity of the liver(16). These observations evidenced an alternative mechanism of action of this drug that may be relevant to cholestasis and lead us to investigate the impact of NorUDCA on SIRT1 expression during cholestasis.

Our results show that NorUDCA significantly reduced SIRT1 expression in SIRT^{oe} mice during cholestasis after BDL, both at the gene transcript (Suppl. Fig 12A) and protein level (Fig 8A, B, Suppl 12B). Lower SIRT1 expression correlated with higher FXR expression in NorUDCA/BDL/SIRT^{oe} mice compared to BDL/SIRT^{oe} (Fig 8C, Suppl 12C). Reduced bile acid pool size was detected in livers from NorUDCA/SIRT^{oe} mice after BDL compared to BDL/SIRT^{oe} animals (Suppl Fig 8D). These changes correlated with an obvious improvement in liver parenchyma status in NorUDCA/SIRT^{oe} mice after BDL as evidenced by H&E staining (Fig 8D), determination of serum liver injury markers (Suppl Fig 12 E), and reduced apoptotic cell death (Fig 8E, F, Suppl. Fig 12F). Finally, NorUDCA/SIRT^{oe} mice showed a significant attenuation of the ductular reaction (Fig 8G) and fibrogenesis (Fig 8H, Suppl Fig 12G) after BDL compared to SIRT^{oe} animals.

Notably, analysis of livers from Mdr2$^{-/-}$ mice confirmed that the described attenuation of the cholestatic phenotype exerted by NorUDCA treatment(4) associates with the reduction of SIRT1 protein expression and nuclear localisation (Suppl Fig 12 H, I).

Taken together, our results support the importance of preserving the fine tuning of SIRT1 expression to protect the liver from cholestasis-induced parenchymal injury.

DISCUSSION

In this study, we provide evidence that SIRT1 is upregulated in the liver during human and murine cholestasis, and that it actively contributes to liver damage in this disease context.

Our results showing that SIRT1 expression is increased in livers from both PBC and PSC cholestatic patients regardless of the disease aetiology, suggest that the upregulation of SIRT1 may be related to the accumulation of bile acids in the liver occurring during obstructive cholestasis. Previous studies evidenced that UDCA and TUDCA induce SIRT1 expression(27, 28), whereas low doses (10-50μM) of unconjugated species had no effect in modulating SIRT1(28). Here we show that primary, conjugated and secondary bile acids (at a dose of 125μM), significantly induce the expression of SIRT1 in human liver THLE cells and in mouse primary hepatocytes, whereas no effect was observed at lower concentrations, supporting that the dosage of bile acids is crucial to regulate SIRT1. Different doses of bile acids have a differential impact on hepatocyte physiology; while low concentrations of bile acids (10-50μM) act as signalling molecules, higher doses (from 50μM to 200μM) have a pro-apoptotic action(29-32). In accordance, our results show that upregulation of SIRT1 expression in response to bile acids (125μM) correlates with apoptotic cell death in primary hepatocytes isolated from WT mice. Importantly, we found that the inhibition of apoptosis had no impact on the upregulation of SIRT1, supporting that SIRT1 is upstream of the apoptotic response. Our additional studies confirmed the pro-apoptotic implication of SIRT1 upregulation in hepatocytes, as apoptosis was further increased in SIRT1 overexpressing-hepatocytes in response to bile acids, whereas it was significantly reduced in SIRT1 depleted hepatocytes compared to WT cells.

Further mechanistic *in vitro* studies pointed to the crosstalk regulation of SIRT1 and AMPK, which is essential to mediate bile acid-induced cell death. Our results are in line with those in previous work showing that AMPK activation by metformin aggravated liver injury during xenobiotic-induced cholestasis, via mechanisms involving impaired FXR signaling(33) and support the relevance of the SIRT1/AMPK axis in mediating bile acids-induced cell death.

SIRT1 and AMPK are well known metabolic regulators activated in response to metabolic challenges including the decrease in cell energy levels (e.g. during starvation/fasting)(23, 24). During cholestasis, the disruption of the flux of bile acids to the intestine contributes to deficient lipid absorption that overall impacts on the metabolic/energy status of the liver. Importantly, work from Moustafa *et al* (34) showed that restoration of lipid metabolism in Mdr2$^{-/-}$ mice after NorUDCA feeding or high fat diet feeding protected the liver from cholestatic-liver injury, pointing to the beneficial impact of increasing energy load during cholestasis. We propose that during cholestasis, the metabolic challenge involving lower nutrient/energy availability, in addition to the increase bile acid load, contribute to the up-regulation of SIRT1 and the subsequent liver damage. Supporting this, our *in vitro* studies show that serum-supplementation to the culture media associated with reduced SIRT1 and AMPK activation and lower apoptosis in response to bile acids in comparison to starved hepatocytes. While further work investigating the *in vivo* activation of AMPK during cholestasis is required, our *in vitro* studies point to a role for AMPK in regulating SIRT1 and detrimental activity during cholestasis. Collectively, our results and those previously published(33, 34) highlight the metabolic characteristic of cholestatic disease.

To gain further insight into the biological relevance of SIRT1 regulation during cholestasis, we performed BDL and fed SIRT1 overexpressing mice with a 0.1%DDC diet that showed exacerbated parenchymal liver injury when compared to WT animals. Additional *in vivo* studies showed that the reduction, but not complete inhibition, of SIRT1 expression in the liver had a therapeutic potential to improve liver parenchyma status during cholestasis.

Thus, attenuation of liver injury in BDL/SIRT^{oe} mice after NorUDCA treatment correlated with a reduction of SIRT1 expression, while hepatocyte-targeted SIRT1 depletion in SIRT$^{hep-/-}$ mice lead to a transient improvement in liver function that was offset at later stages after BDL. The regulation of FXR by SIRT1 may represent a key mechanism mediating these outcomes.

FXR is the main regulator of bile acid homeostasis. During cholestasis, FXR signalling mediates an adaptive response aiming to reduce bile acid pool size by inhibiting bile acid synthesis and modulating their transport(35). FXR currently represents a promising target for novel therapeutic approaches to treat human cholestatic disease(7-9). The regulation of FXR involves a dynamic deacetylation process coordinated by SIRT1(14), and is needed for FXR-DNA binding and target gene transcription, while the same process regulates FXR proteasomal degradation(14). In accordance, we previously described that FXR was reduced in SIRT1 overexpressing mice(16). Here, we provide further evidence of the relevance of SIRT1/FXR signalling during cholestasis. Thus, whereas SIRT1 overexpression reduced FXR-signalling, attenuation of SIRT1 after NorUDCA treatment efficiently restored FXR expression after BDL. Interestingly, we also observed reduced FXR signalling in SIRT1$^{hep-/-}$ mice, as described in previous studies(36) that associated with a transient reduction of liver injury after BDL, suggesting that depletion of SIRT1/FXR in hepatocytes may protect the liver at early stages of obstructive cholestasis. This is supported by previous studies(11, 35, 37) including work from Wagner et al., showing that whole body-FXR deficient mice had lower intrabiliary pressure after BDL overall relating to less bile infarcts and attenuated liver damage after BDL(11). Similarly, we found reduced ductular reaction at early stages of cholestasis in both SIRT^{oe} and SIRT1$^{hep-/-}$ mice though differences were not statistical significant in the latter and became comparable to WT mice at later stages after BDL.

As cholestatic disease progresses, cholangiocytes lose their proliferative capacity in advanced disease, contributing to bile duct loss (ductopenia)(3). Our results point to the

contribution of SIRT1 to this process and support the previously described ability of mild SIRT1 overexpression to inhibit the proliferative effect of growth factors like PGRN *in vitro*(38).

Ultimately, the apparent differences in the severity of the damaging phenotype observed in SIRT1 overexpressing mice when compared to $SIRT1^{hep-/-}$ mice, despite the similarly attenuation of FXR signalling, support that apoptotic cell death associated with increased SIRT1 expression play a key role in contributing to liver injury during cholestasis.

Several studies using NorUDCA treatment in murine models of cholestasis(4, 25, 39, 40) and a recently conducted phase II human clinical trial(5), support the benefits of this drug as a treatment option for cholestatic patients(5, 41). In our present work, we show that NorUDCA modulates SIRT1 expression in two alternative models of cholestasis; in $SIRT^{oe}$ mice after BDL and in $Mdr2^{-/-}$ mice. Importantly, our results show that only the modulation of SIRT1 exerted by NorUDCA, but not the complete depletion (as in our hepatocyte-KO mice), preserved FXR signalling and overall liver function after BDL, emphasizing the relevance of maintaining fine-tuned SIRT1 expression to protect the liver during cholestasis. In our studies, we cannot discern whether SIRT1 regulation is a mere consequence of the reduced bile acid pool size in the liver or is a direct effect of NorUDCA on SIRT1. As discussed, other factors like the restoration of liver energy metabolism upon NorUDCA treatment(34) may also impact on SIRT1 regulation.

Our observations are relevant to recent studies that propose the use of SIRT1 activators to counteract murine cholestasis after cholic acid feeding in mice(28). It's worth noting that after bile acid feeding, SIRT1 was differently regulated than during human and murine obstructive cholestasis, where SIRT1 expression is significantly elevated. Although it is out of the scope of our current study to resolve the differential SIRT1 expression during cholic acid feeding and after BDL, previous studies have revealed marked differences between these two experimental models(11, 42). For example, cholic acid feeding regulates

intestinal pathways that feedback to control bile acid metabolism in the liver in a different way to that during obstructive cholestasis, which involves the absence of bile acids in the intestine. Hence, cholic acid feeding commonly results in inhibition of Cyp7A1(43), likely mediated by intestinal-derived feedback mechanisms involving activation of ileal FXR(44), whereas obstructive cholestasis after BDL results in initial reduction (Fig 4, 5) but later recovery of Cyp7A1 expression and bile acid synthesis(44). Furthermore, treatment with SIRT1 activators in cholic acid-fed mice had no impact on SIRT1 gene expression and protein expression was only modestly induced after treatment, rendering a SIRT1 expression comparable to that found at basal homeostatic conditions(28). These observations ultimately support our conclusions underscoring the importance of maintaining a fine-tuned SIRT1 expression in the liver to counteract cholestasis.

In summary, our work raises awareness that the expression levels of SIRT1 should be considered when designing therapeutic strategies to treat cholestasis, which should aim to the attenuation, though not complete inhibition, of SIRT1. Overall, our results underline the critical relevance of maintaining the fine-tuning of SIRT1 expression to preserve liver health.

REFERENCES

1. Dyson JK, Hirschfield GM, Adams DH, Beuers U, Mann DA, Lindor KD, Jones DE. Novel therapeutic targets in primary biliary cirrhosis. Nat Rev Gastroenterol Hepatol 2015;12:147-158.

2. Eaton JE, Talwalkar JA, Lazaridis KN, Gores GJ, Lindor KD. Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management. Gastroenterology 2013;145:521-536.

3. Hirschfield GM, Heathcote EJ, Gershwin ME. Pathogenesis of cholestatic liver disease and therapeutic approaches. Gastroenterology 2010;139:1481-1496.

4. Halilbasic E, Fiorotto R, Fickert P, Marschall HU, Moustafa T, Spirli C, Fuchsbichler A, et al. Side chain structure determines unique physiologic and therapeutic properties of norursodeoxycholic acid in Mdr2-/- mice. Hepatology 2009;49:1972-1981.

5. Fickert P, Hirschfield GM, Denk G, Marschall HU, Altorjay I, Farkkila M, Schramm C, et al. norUrsodeoxycholic acid improves cholestasis in primary sclerosing cholangitis. J Hepatol 2017;67:549-558.

6. Beuers U, Trauner M, Jansen P, Poupon R. New paradigms in the treatment of hepatic cholestasis: from UDCA to FXR, PXR and beyond. J Hepatol 2015;62:S25-37.

7. Hirschfield GM, Mason A, Luketic V, Lindor K, Gordon SC, Mayo M, Kowdley KV, et al. Efficacy of obeticholic acid in patients with primary biliary cirrhosis and inadequate response to ursodeoxycholic acid. Gastroenterology 2015;148:751-761 e758.

8. Kowdley KV, Luketic V, Chapman R, Hirschfield GM, Poupon R, Schramm C, Vincent C, et al. A randomized trial of obeticholic acid monotherapy in patients with primary biliary cholangitis. Hepatology 2017.

9. Nevens F, Andreone P, Mazzella G, Strasser SI, Bowlus C, Invernizzi P, Drenth JP, et al. A Placebo-Controlled Trial of Obeticholic Acid in Primary Biliary Cholangitis. N Engl J Med 2016;375:631-643.

10. Gomez-Ospina N, Potter CJ, Xiao R, Manickam K, Kim MS, Kim KH, Shneider BL, et al. Mutations in the nuclear bile acid receptor FXR cause progressive familial intrahepatic cholestasis. Nat Commun 2016;7:10713.

11. Wagner M, Fickert P, Zollner G, Fuchsbichler A, Silbert D, Tsybrovskyy O, Zatloukal K, et al. Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice. Gastroenterology 2003;125:825-838.

12. Claudel T, Zollner G, Wagner M, Trauner M. Role of nuclear receptors for bile acid metabolism, bile secretion, cholestasis, and gallstone disease. Biochim Biophys Acta 2011;1812:867-878.

13. Cariello M, Piccinin E, Garcia-Irigoyen O, Sabba C, Moschetta A. Nuclear receptor FXR, bile acids and liver damage: Introducing the progressive familial intrahepatic cholestasis with FXR mutations. Biochim Biophys Acta 2018;1864:1308-1318.

14. Kemper JK, Xiao Z, Ponugoti B, Miao J, Fang S, Kanamaluru D, Tsang S, et al. FXR acetylation is normally dynamically regulated by p300 and SIRT1 but constitutively elevated in metabolic disease states. Cell metabolism 2009;10:392-404.

15. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nat Rev Mol Cell Biol 2012;13:225-238.

16. Garcia-Rodriguez JL, Barbier-Torres L, Fernandez-Alvarez S, Gutierrez-de Juan V, Monte MJ, Halilbasic E, Herranz D, et al. SIRT1 controls liver regeneration by regulating bile acid metabolism through farnesoid X receptor and mammalian target of rapamycin signaling. Hepatology 2014;59:1972-1983.

17. Guarente L. Calorie restriction and sirtuins revisited. Genes Dev 2013;27:2072-2085.

18. Herranz D, Munoz-Martin M, Canamero M, Mulero F, Martinez-Pastor B, Fernandez-Capetillo O, Serrano M. Sirt1 improves healthy ageing and protects from metabolic syndrome-associated cancer. Nature communications 2010;1:3.

19. Fang Y, Nicholl MB. Sirtuin 1 in malignant transformation: friend or foe? Cancer Lett 2011;306:10-14.

20. Chen HC, Jeng YM, Yuan RH, Hsu HC, Chen YL. SIRT1 promotes tumorigenesis and resistance to chemotherapy in hepatocellular carcinoma and its expression predicts poor prognosis. Ann Surg Oncol 2012;19:2011-2019.

21. Wasik U, Milkiewicz M, Kempinska-Podhorodecka A, Milkiewicz P. Protection against oxidative stress mediated by the Nrf2/Keap1 axis is impaired in Primary Biliary Cholangitis. Sci Rep 2017;7:44769.

22. Fickert P, Fuchsbichler A, Wagner M, Zollner G, Kaser A, Tilg H, Krause R, et al. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2004;127:261-274.

23. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 2005;434:113-118.

24. Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y. AMPK and SIRT1: a long-standing partnership? American journal of physiology. Endocrinology and metabolism 2010;298:E751-760.

25. Fickert P, Wagner M, Marschall HU, Fuchsbichler A, Zollner G, Tsybrovskyy O, Zatloukal K, et al. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2006;130:465-481.

26. Beraza N, Malato Y, Vander Borght S, Liedtke C, Wasmuth HE, Dreano M, de Vos R, et al. Pharmacological IKK2 inhibition blocks liver steatosis and initiation of non-alcoholic steatohepatitis. Gut 2008;57:655-663.

27. Castro RE, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, Rodrigues CM. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. Journal of hepatology 2013;58:119-125.

28. Kulkarni SR, Soroka CJ, Hagey LR, Boyer JL. Sirtuin 1 activation alleviates cholestatic liver injury in a cholic acid-fed mouse model of cholestasis. Hepatology 2016;64:2151-2164.

29. Li Y, Tang R, Leung PSC, Gershwin ME, Ma X. Bile acids and intestinal microbiota in autoimmune cholestatic liver diseases. Autoimmun Rev 2017;16:885-896.

30. Jansen PL, Ghallab A, Vartak N, Reif R, Schaap FG, Hampe J, Hengstler JG. The ascending pathophysiology of cholestatic liver disease. Hepatology 2017;65:722-738.

31. Guicciardi ME, Gores GJ. Bile acid-mediated hepatocyte apoptosis and cholestatic liver disease. Dig Liver Dis 2002;34:387-392.

32. Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. Compr Physiol 2013;3:977-1010.

33. Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, et al. Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. J Clin Invest 2014;124:1037-1051.

34. Moustafa T, Fickert P, Magnes C, Guelly C, Thueringer A, Frank S, Kratky D, et al. Alterations in lipid metabolism mediate inflammation, fibrosis, and proliferation in a mouse model of chronic cholestatic liver injury. Gastroenterology 2012;142:140-151 e112.

35. Yuan ZQ, Li KW. Role of farnesoid X receptor in cholestasis. J Dig Dis 2016;17:501-509.

36. Purushotham A, Xu Q, Lu J, Foley JF, Yan X, Kim DH, Kemper JK, et al. Hepatic deletion of SIRT1 decreases hepatocyte nuclear factor 1alpha/farnesoid X receptor signaling and induces formation of cholesterol gallstones in mice. Mol Cell Biol 2012;32:1226-1236.

37. Stedman C, Liddle C, Coulter S, Sonoda J, Alvarez JG, Evans RM, Downes M. Benefit of farnesoid X receptor inhibition in obstructive cholestasis. Proc Natl Acad Sci U S A 2006;103:11323-11328.

38. Frampton G, Ueno Y, Quinn M, McMillin M, Pae HY, Galindo C, Leyva-Illades D, et al. The novel growth factor, progranulin, stimulates mouse cholangiocyte proliferation via sirtuin-1-mediated inactivation of FOXO1. Am J Physiol Gastrointest Liver Physiol 2012;303:G1202-1211.

39. Denk GU, Maitz S, Wimmer R, Rust C, Invernizzi P, Ferdinandusse S, Kulik W, et al. Conjugation is essential for the anticholestatic effect of NorUrsodeoxycholic acid in taurolithocholic acid-induced cholestasis in rat liver. Hepatology 2010;52:1758-1768.

40. Fickert P, Pollheimer MJ, Silbert D, Moustafa T, Halilbasic E, Krones E, Durchschein F, et al. Differential effects of norUDCA and UDCA in obstructive cholestasis in mice. Journal of hepatology 2013;58:1201-1208.

41. Wagner M, Trauner M. Recent advances in understanding and managing cholestasis. F1000Res 2016;5.

42. Zollner G, Fickert P, Fuchsbichler A, Silbert D, Wagner M, Arbeiter S, Gonzalez FJ, et al. Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. J Hepatol 2003;39:480-488.

43. Li-Hawkins J, Gafvels M, Olin M, Lund EG, Andersson U, Schuster G, Bjorkhem I, et al. Cholic acid mediates negative feedback regulation of bile acid synthesis in mice. J Clin Invest 2002;110:1191-1200.

44. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab 2005;2:217-225.

FIGURE LEGENDS

Figure 1. SIRT1 is highly expressed in livers from cholestatic PBC and PSC patients and is induced in response to bile acids *in vitro*.

(A) qPCR analysis of SIRT1 expression in liver samples from healthy individuals n=5, PBC n=10 and PSC n=10 and (B) IHC using an anti-SIRT1 Ab in liver sections from cholestatic patients compared to healthy donors showing increased gene and protein expression and protein nuclear localization of SIRT1 in hepatocytes and cholangiocytes during cholestasis (original magnification 10x) with (C) quantification of positively stained nuclei. Healthy individuals n=4, PBC n=9, PSC n=5. (D) qPCR analysis of SIRT1 expression in THLE2 cells cultured for 3h with CA, GCA, CDCA, DCA and at the doses indicated. (Values are mean ± SEM; *in vitro* experiments were performed three times in triplicate; **P* <0.05, ***P* <0.01).

Figure 2. SIRT1 expression is up-regulated during surgically and genetically-induced murine cholestasis.

(A) qPCR analysis of SIRT1 expression in livers from WT mice at different time points after BDL showing upregulation during cholestasis. (B) Western blot analysis on liver nuclear extracts from WT mice and (C) IHC on liver sections and (D) further quantification of SIRT1-positive nuclei after BDL indicating increased SIRT1 expression and nuclear localization during cholestasis. (E) IHC in liver sections of WT and $Mdr2^{-/-}$ mice and (F) quantification of SIRT1-positive nuclei. (Values are mean ± SEM. n≥5 animals/time point; ***P* <0.01).

Figure 3. Overexpression of SIRT1 leads to increased parenchymal injury and fibrogenesis in mice after BDL.

(A) Profiles of blood liver injury markers detected in WT and SIRT^{oe} animals and (B) H&E staining of liver sections from WT and SIRT^{oe} animals after BDL showing profuse liver damage in SIRT^{oe} mice. (C) TUNEL assay on liver sections showing increased presence of apoptotic hepatocytes in SIRT^{oe} mice compared to WT after BDL. (D) Caspase-3 activity was determined in primary hepatocytes isolated from WT and SIRT^{oe} mice and cultured in the presence of CDCA and DCA. (E) FACS analysis on liver isolated immune cells and (F) qPCR analyses of inflammation markers showed increased presence of macrophages and increased proinflammatory response in SIRT^{oe} mice. (G) Liver fibrogenesis was characterised by Sirius Red staining on liver sections (left panels) and αSMA IHC (right panels) from mice after BDL followed by morphometric quantification using Frida software expressed in % of positive staining per power field (ppf). (Images are representative of n≥5 animals/time point; Values are mean ± SEM. **P* <0.05, ***P* <0.01, *** *P* <0.001 [WT *vs* SIRT^{oe}]).

Figure 4. Overexpression of SIRT1 correlates with lower presence and activity of FXR and higher accumulation of bile acids in the liver.

(A) Western blot of whole protein extracts using FXR Ab and further quantification using Image Lab software (BioRad) showing reduced protein presence in SIRT^{oe} mice. (B) Gene expression analysis of SHP and Cyp7a1 by qPCR in livers 3 and 7 days after BDL. (C) Quantification of bile acid pool size in livers from WT and SIRT mice after BDL by HPLC. (D) qPCR analysis of bile acids transporters after BDL (Values are mean ± SEM. n≥5 animals/time point; **P* <0.05, ***P* <0.01 [WT *vs* SIRT^{oe}]).

Figure 5. SIRT1 overexpression correlates with attenuation of cholangiocyte proliferation (A) IHC using an anti-CK19 Ab in paraffin-embedded liver sections at different timepoints after BDL and (B) further quantification using Frida software, expressed in % of positive staining ppf (original magnification 10x) showing milder ductular reaction in SIRT mice compared to WTs. (C) Determination of caspase-3 activity in response to CDCA on normal mouse cholangiocytes (NMC) transfected with control c-Flag pcDNA3 (Ctrl) or pCruzHA SIRT1 plasmid DNA to induce overexpression of SIRT1 ($SIRT^{oe}$). (D) qPCR analysis of cell cycle-related gene expression in Ctrl and $SIRT^{oe}$ transfected NMC showing lower proliferation in the presence of growth factors (EGF) 36h after transfection. (E) FACS analysis of PI stained NMC confirming lower numbers of cells in S phase after SIRT1 overexpression compared to control transfected cells. (F) Representative histograms after FACS analysis of NMC transfected cells in culture. (Values are mean ± SD. n = 5 animals/time point; *in vitro* experiments were performed three times in triplicate. **P* <0.05, ***P* <0.01 [WT *vs* SIRT]; [Ctrl *vs* $SIRT1^{oe}$]).

Figure 6. Hepatocyte-specific SIRT1 depletion has a transient effect on protecting the liver from cholestatic-injury via modulating apoptotic cell death whereas has no impact on liver inflammation, ductular reaction and fibrosis.

(A) Levels of serum liver damage markers of WT and $SIRT^{hep-/-}$ animals and (B) H&E staining of liver sections from WT and $SIRT^{hep-/-}$ animals after BDL showing transient improvement of the damaging liver phenotype in $SIRT^{hep-/-}$ mice compared to WT. (C) TUNEL assay on liver sections showing decreased presence of apoptotic hepatocytes in $SIRT^{hep-/-}$ mice compared to WT after BDL. (D) Quantification of caspase-3 activity and in CDCA and DCA treated primary hepatocytes isolated from WT and $SIRT^{hep-/-}$ mice showing a decrease apoptosis in $SIRT^{hep-/-}$ mice, indicating an increase in necrosis. (E) FACS analysis on liver isolated immune cells and (F) ELISA on liver extracts showed comparable

presence of macrophages and cytokine production in SIRT$^{hep-/-}$ and WT mice. (G) Liver fibrogenesis was characterised by Sirius Red staining on liver sections (left panels) and aSMA IHC (right panels) from mice after BDL followed by quantification using Frida software expressed in % of positive staining per power field (ppf). All images at original magnification 10x Values are mean ± SEM. n≥5 animals/time point; *in vitro* experiments were performed three times in triplicate; **P* <0.05, ***P* <0.01 [WT *vs* SIRT$^{hep-/-}$]).

Figure 7. Hepatocyte-specific SIRT1 depletion associates with reduced FXR expression and signalling after BDL and comparable bile acid transporters expression than WT littermates.

(A) Western blot of whole protein extracts and further quantification using ImageLab software showing reduced FXR in SIRT$^{hep-/-}$ mice after BDL (B) Gene expression analysis of SHP and Cyp7a1 and (C) bile acid transporters by qPCR in livers 3 and 7days after BDL. (D) Quantification of bile acid pool size in livers from WT and SIRT$^{hep-/-}$ mice after BDL by MS-HPLC. Values are mean ± SEM. n≥5 animals/time point; ***P* <0.01 [WT *vs* SIRT$^{hep-/-}$]).

Figure 8. NorUDCA lowers SIRT1 expression leading to restored FXR signalling, improved liver parenchyma status and reduced apoptosis, ductular reaction and fibrogenesis after BDL.

(A) IHC in liver sections using a SIRT1 Ab and (B) western blot analysis showing reduction SIRT1 but (C) sustained FXR protein expression in NorUDCA/SIRT^{oe} mice after BDL (D) H&E staining of liver sections, (E) quantification of caspase-3 activity on liver protein lysates (F) and TUNEL assay on liver sections from SIRT^{oe} and NorUDCA/SIRT^{oe} mice after BDL confirmed attenuation of parenchymal injury in the latter. (G) CK19 IHC as well as (H) Sirius Red staining and aSMA IHC on liver sections all followed by morphometric analyses

confirmed the beneficial impact of NorUDCA in BDL/SIRT^{oe} mice. All images at original magnification 10x or 20x (SIRT1). Values are mean ± SEM. n≥5 animals/time point; *P <0.05, **P <0.01 [SIRT *vs* NorUDCA/SIRT].

Acknowledgements

We want to thank Prof Manuel Serrano for kindly providing us with the SIRT1 overexpressing mice. We thank Prof. Mark Wilkinson (Norwich and Norfolk University Hospital Tissue Bank) for kindly providing us with human liver tissue slides. We want to thank Clare Sansom and Ian Young for editing the MS.

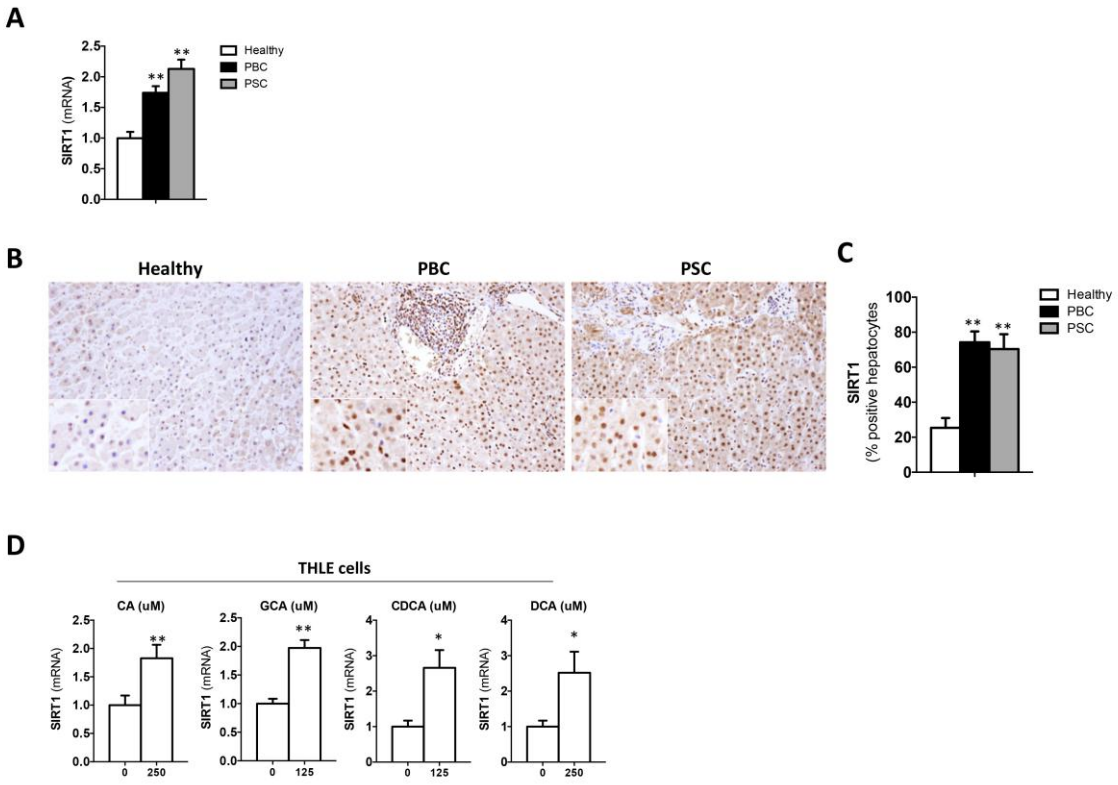

Figure 1

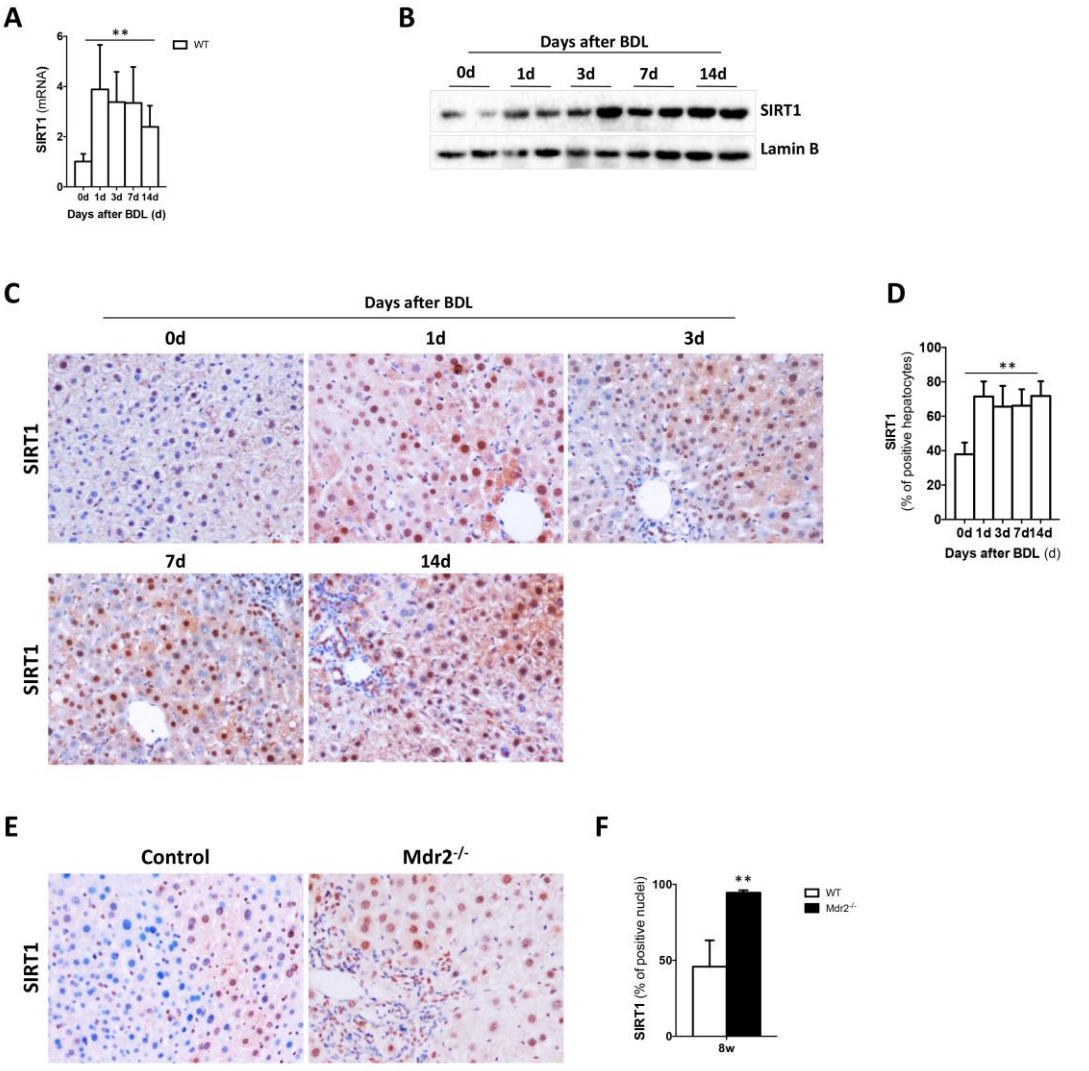

Figure 2

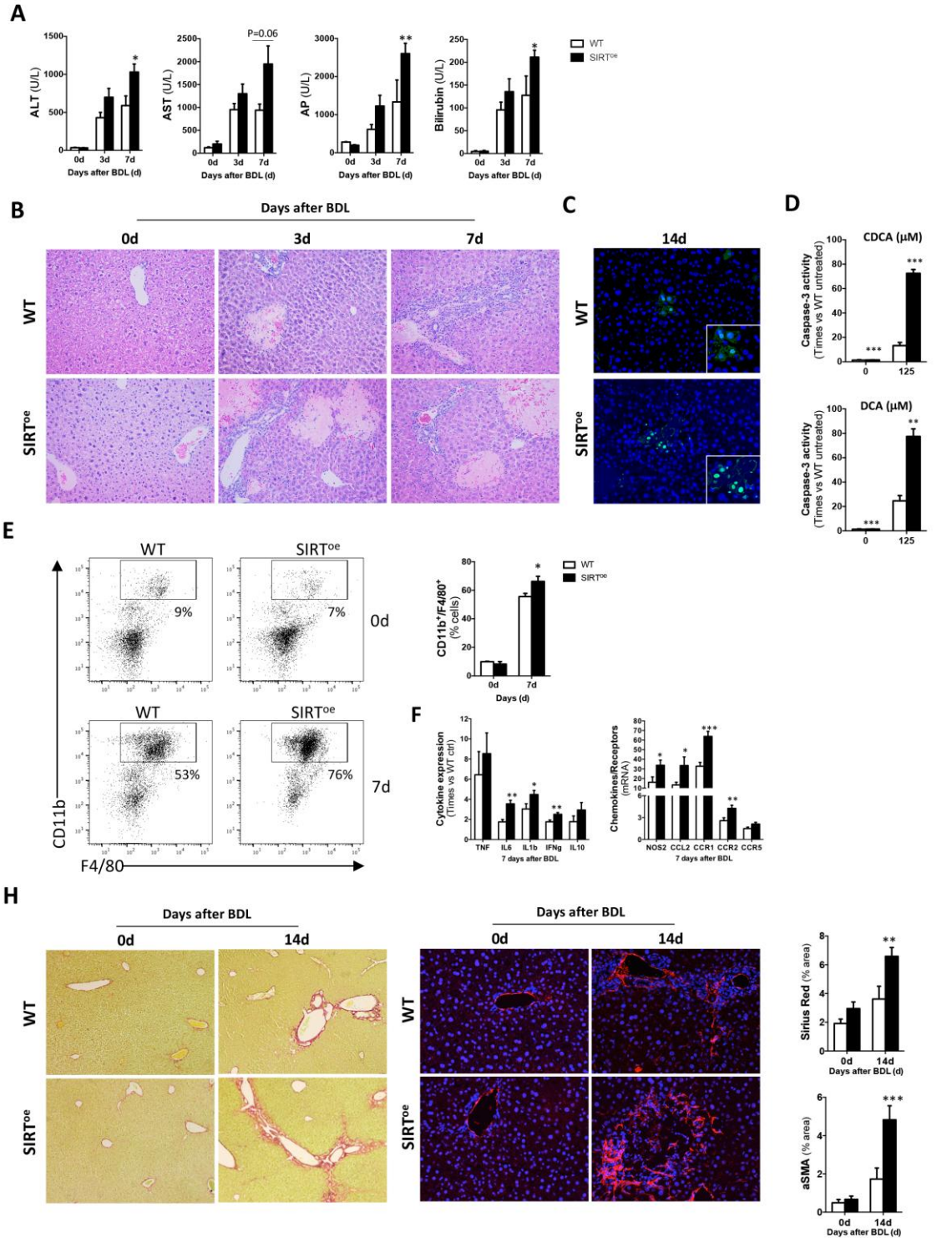

Figure 3

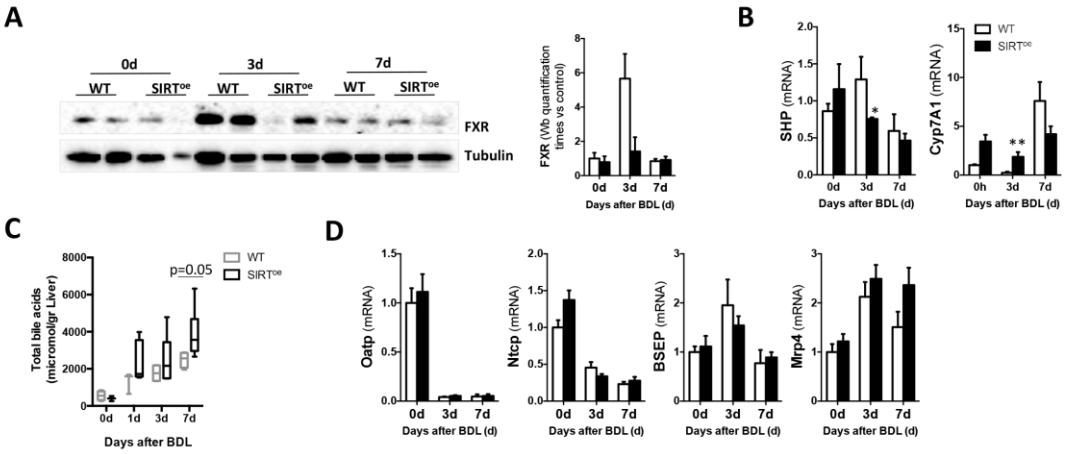

Figure 4

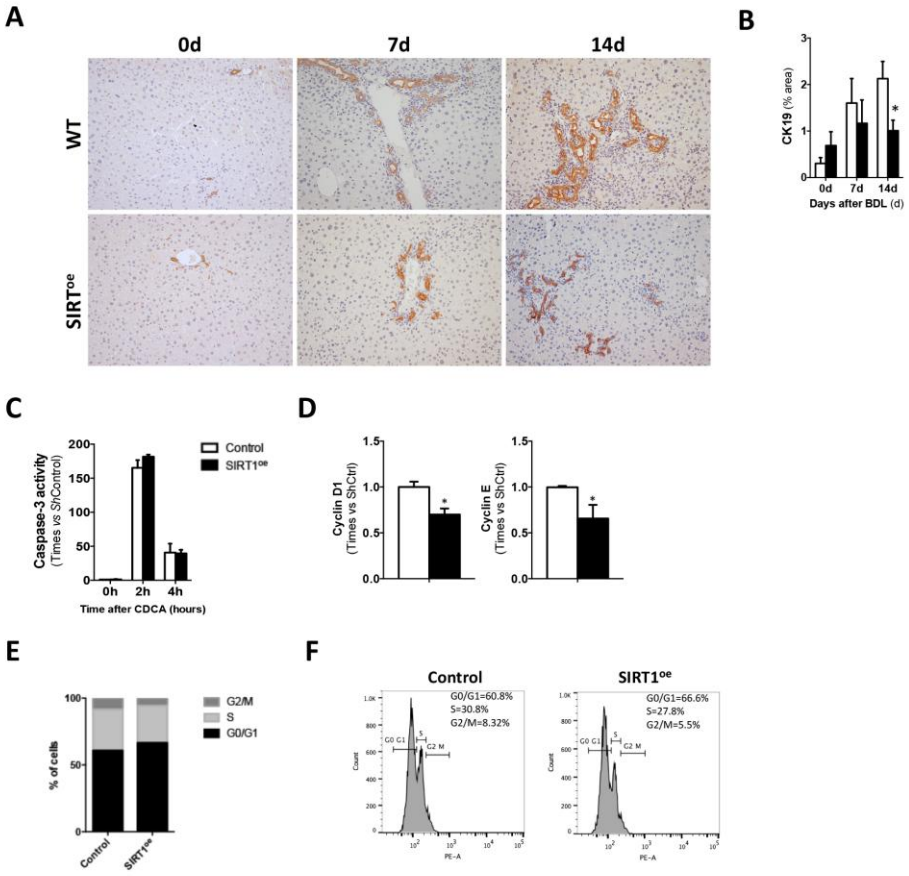

Figure 5

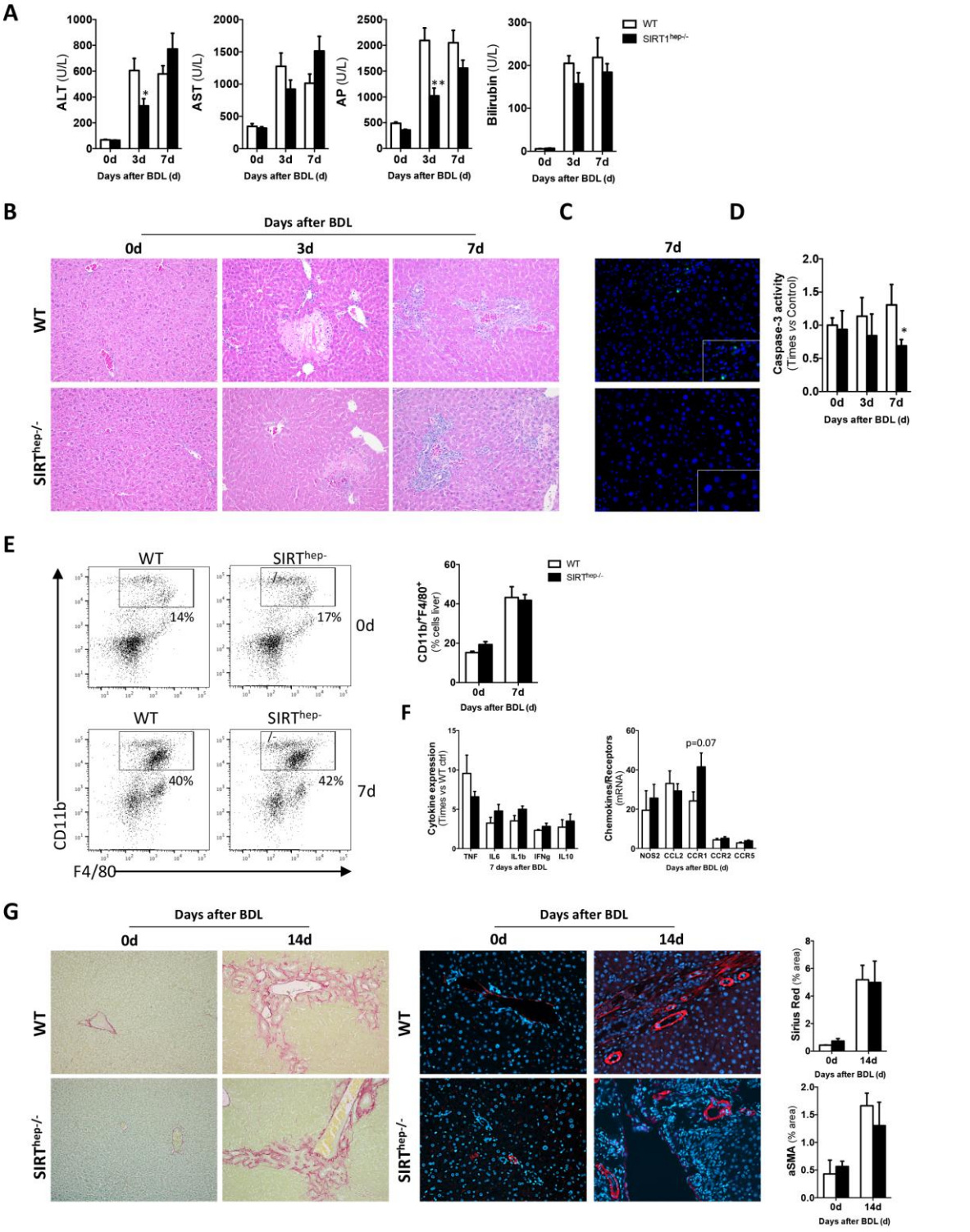

Figure 6

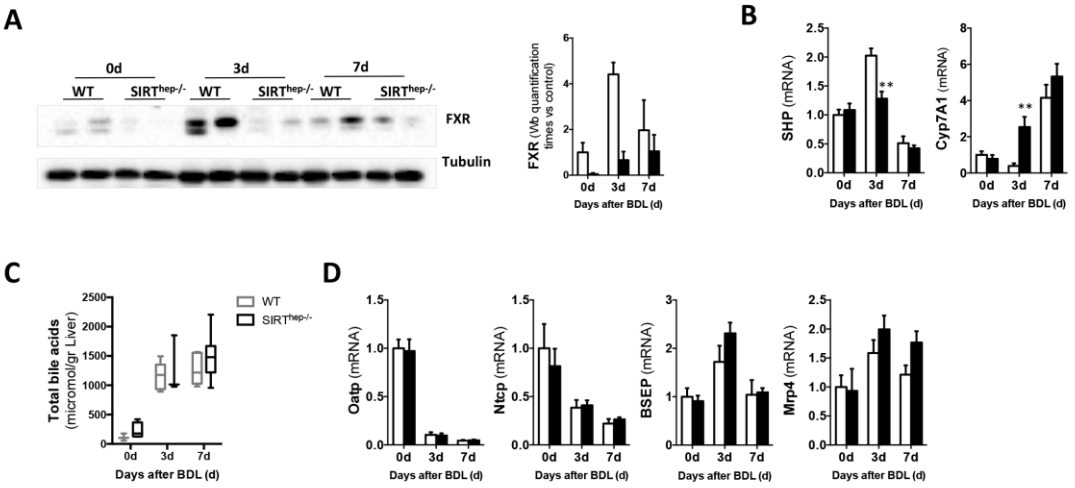

Figure 7

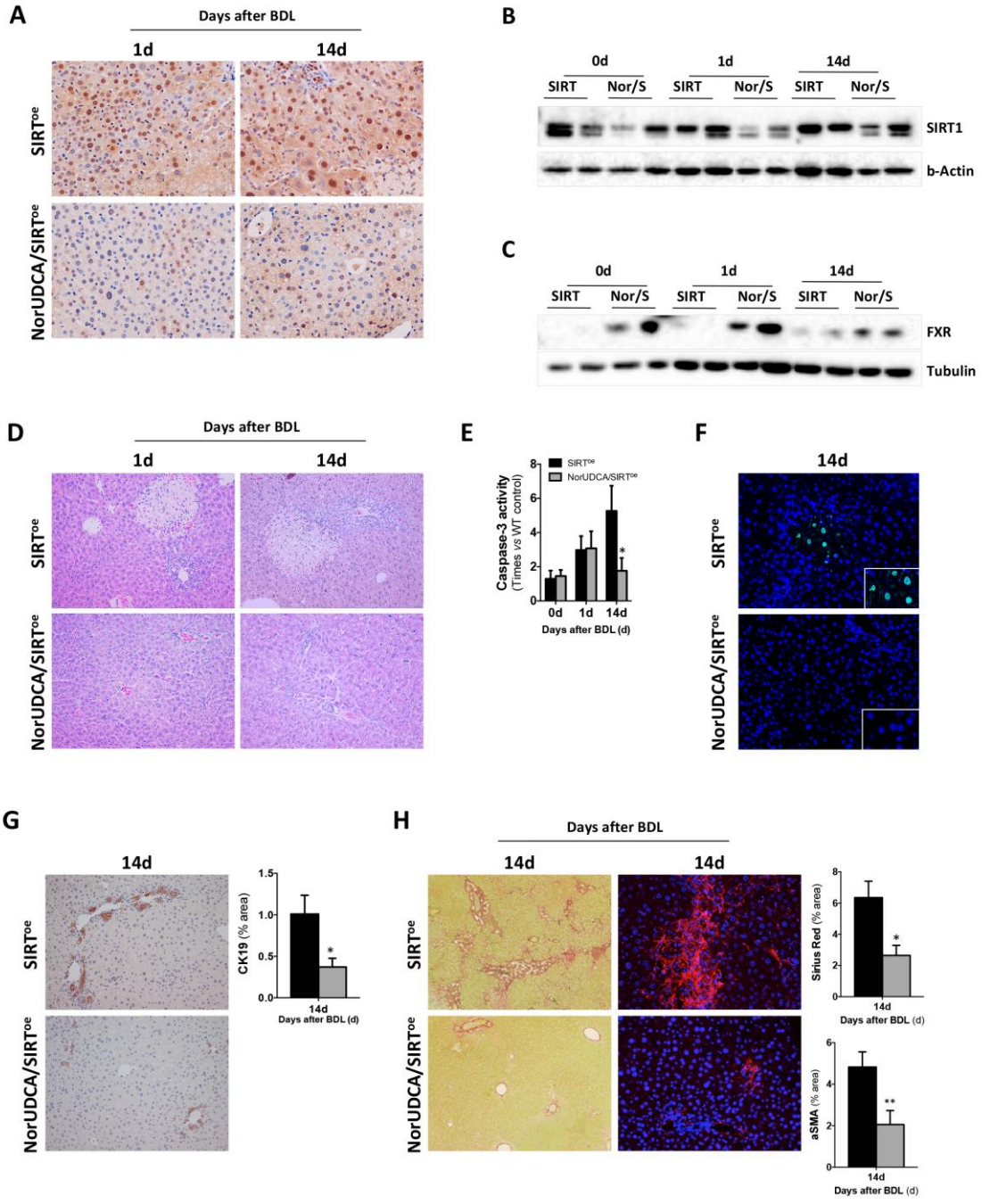

Figure 8