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LKB1 IS REQUIRED FOR HEPATIC BILE ACID TRANSPORT AND CANALICULAR MEMBRANE INTEGRITY IN MICE

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Synopsis

LKB1 is a “master” protein kinase implicated in the regulation of metabolism, cell proliferation, cell polarity and tumorigenesis. However, the long-term role of LKB1 in hepatic function is unknown. Here it is shown that hepatic LKB1 plays a key role in liver cellular architecture and metabolism. We report that liver-specific deletion of LKB1 in mice leads to defective canaliculi and bile duct formation, causing impaired bile acid clearance and subsequent accumulation of bile acids in serum and liver. Concomitant with this, it was found that the majority of bile salt export pump (BSEP) was retained in intracellular pools rather than localized to the canalicular membrane in hepatocytes from liver-specific LKB1 knockout (LLKB1KO) mice. Together, these changes resulted in toxic accumulation of bile salts, reduced liver function and failure to thrive. Additionally, circulating low-density lipoprotein (LDL)- and unesterified-cholesterol levels were increased in LLKB1KO mice with an associated alteration in erythrocyte morphology and development of hyperbilirubinemia. These results indicate that LKB1 plays a critical role in bile acid homeostasis and that lack of LKB1 in the liver results in cholestasis. These findings indicate a novel, key role for LKB1 in development of hepatic morphology and membrane targeting of canalicular proteins.

Key words: bile salt export pump, ABCB11, hyperbilirubinemia, cholestasis, AMPK, polarity.

Abbreviations: AMPK, AMP-activated protein kinase; BSEP (bile salt export pump); LDL, low-density lipoprotein; MRP, multi-drug resistance protein; NTCP, Na⁺taurocholate co-transporting protein; qRT-PCR, quantitative real-time polymerase chain reaction; wt, wild type; LLKB1KO, liver specific LKB1 knockout.

Introduction

LKB1 encodes an evolutionary conserved serine/threonine protein kinase that was originally identified as a tumor suppressor since inactivating mutations in LKB1 in humans cause Peutz-Jeghers Syndrome, [1, 2]. More recently, LKB1 has been shown to act upstream of AMP-activated protein kinase (AMPK) [3-5] and 12 AMPK-related kinases [6]. LKB1 phosphorylates a conserved threonine residue within the T-loop of these kinases, which is essential for their activation [7]. Activation of AMPK by LKB1 under conditions of energy depletion results in down-regulation of energy consuming pathways, and up-regulation of ATP producing pathways. Much less is understood regarding the roles of the AMPK-related kinases, although there is evidence to suggest that the MARK [8] and BRSK [9, 10] sub-families play roles in determining cell polarity. Information concerning the physiological function of the remaining AMPK-related kinases is extremely limited [11].

Germline deletion of LKB1 leads to an embryonic-lethal phenotype with mice dying from a variety of vascular and placental defects demonstrating a key developmental role [12]. Tissue specific deletion of LKB1 has been investigated in several mouse models [13-15] with phenotypes affecting varying aspects of cell morphology and organ dysfunction. Taken together these findings suggest that LKB1 plays a key role in integrating cell and tissue morphology with metabolic function.

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The liver has many metabolic functions including bile production together with key regulatory roles in glucose, lipid and xenobiotic metabolism. Bile is the main vehicle by which the body disposes of excess cholesterol by conversion to bile acids and the route used for the excretion of waste products such as bilirubin, a breakdown product of haem. Because high levels of bile acids can cause tissue damage due to their strong detergent properties, their concentrations are tightly regulated by transcriptional control of many genes in a complex feed-back mechanism involving bile acid activation of the Farnesoid X Receptor (FXR) (reviewed by [16]). Following their synthesis in the liver, bile acids are secreted into the bile, stored in the gall bladder and released post-prandially into the small intestine where they play a critical role in the absorption of fat-soluble nutrients. The majority of the bile acids are then returned to the liver via the portal circulation via the apical sodium dependent bile acid transporter (ASBT) in the epithelial cells of the small intestine. Completion of the enterohepatic circulation occurs when the bile acids are returned to the liver mainly by transport via the sodium dependant transporter, NTCP. A major feature of hepatocytes is their marked anatomical polarity, which plays an essential role in their function. For example, the polar nature of hepatocytes allows the efficient vectorial transport of bile acids from the portal blood into the hepatocytes via NTCP and then into the intrahepatic biliary system via the bile salt export pump (BSEP). BSEP is a member of the ATP-binding cassette transporter family and is classified as ABCB11. BSEP is regulated both transcriptionally and post-translationally and mediates canalicular bile formation [17]. Here we show that hepatic LKB1 plays a key role in liver cellular architecture and metabolism. Absence of hepatic LKB1 results in mis-localisation of BSEP, bile duct paucity and impairment of postnatal biliary tree formation. Liver LKB1 knockout (LLKB1KO) mice also display impaired bile acid and lipid metabolism, and die within 4 weeks of age.

Materials and Methods

Generation of mice lacking hepatic LKB1: Production of mice harboring *Lkb1* floxed alleles is described elsewhere [14]. These mice were crossed with *B6.Cg-Tg (Alb-Cre) 21 Mgn/J* transgenic mice harbouring Cre-recombinase under the albumin promoter (Jackson Laboratories Maine USA). All animal studies were performed in accordance to the Animal Scientific Procedures Act. Animals were killed by cervical dislocation, organs rapidly removed and frozen in liquid nitrogen.

Western Blotting: Proteins were resolved on 4-12% gradient gels (Novex, Invitrogen), transferred to polyvinylidene fluoride membranes and probed with antibodies as described in the text. Antibodies were detected by enhanced chemiluminescence (Pierce West Dura kit).

Antibodies: LKB1 sheep antibody raised against residues 24–39 of human LKB1 was a kind gift from Prof. Dario Alessi (University of Dundee). LKB1 mouse monoclonal antibody (Ley37D/G6) and goat anti-radixin antibody was from Santa Cruz Biotechnology Inc. LKB1 rabbit serum [18], rabbit pan AMPK β antibody and sheep anti- α 1 and - α 2 antibodies [19] were as described previously. BSEP and NTCP antibodies were kind gifts from Prof. Bruno Steiger (University Hospital, Zurich)[20, 21], goat antibody to mouse osteopontin (R&D systems), CK19 antibody was from Dako (IS615).

Haemocrit measurement: Blood was collected in haemocrit tubes and centrifuged at 3,000 x g. Packed red cell volume was measured as a ratio of total blood sample volume.

Immune complex kinase assays: LKB1 or AMPK complexes were immunoprecipitated from soluble liver lysates using antibodies bound to either protein A or protein G Sepharose. After extensive washing, kinase activity present in the immune complexes was determined [3]. AMPK

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activity was measured by ^{32}P -phosphate incorporation into the SAMS peptide [22]. LKB1 activity was measured by activation of bacterially expressed AMPK complex ($\alpha 1, \beta 1, \gamma 1$), which was subsequently assayed using the SAMS peptide.

Osmotic fragility test: This was performed as described by Foller et al. [23].

Transmission Electron Microscopy: After glutaraldehyde fixation and processing samples were embedded in araldite. Semi thin sections of 0.5–1 μm were stained with toluidine blue in borax. Ultra-thin sections were stained in uranyl acetate followed by Reynold's lead citrate.

Histology and Immunohistochemistry: Tissue was fixed in formalin saline and processed in paraffin wax. Routine sections were stained with Haematoxylin and Eosin. Immunohistochemical staining was carried out on the Dako Autostainer Plus using a diaminobenzidine-based system to identify antibody binding.

Immunofluorescence microscopy: Livers were fixed in paraformaldehyde and frozen sections used for staining. Appropriate 488-Alexafluor secondary antibodies were used and DAPI used as a nuclear stain.

Liver function tests and metabolite measurements: Serum levels of albumin, alkaline phosphatase, total cholesterol, LDL and HDL cholesterol, bilirubin and triglycerides were determined by the Mouse Biochemistry Laboratories, Cambridge, UK. Assays were measured colourimetrically on a Dade Behring RXL autoanalyser. Esterified and unesterified cholesterol measurements were made using Amplex Red Cholesterol kit (Molecular Probes). Bile acids were measured using TBA Bile acid kit (Sentinel Diagnostics). Bile acids were extracted from livers by homogenization in 10 times (w/v) 70% ethanol.

Isolation of hepatocytes: Hepatocytes were isolated by collagenase perfusion of livers from 14–18 day old, anesthetized mice. After isolation cells were seeded in collagen coated dishes in Medium 199 with Earles and L-glutamine (Gibco) supplemented with UltrosorG (Pall Life Sciences), 1% albumin, 100 nM insulin, 100 nM triiodothyronine (T_3) and 100 nM dexamethasone. After cell attachment, the hepatocytes were cultured for 16–18 h in the absence of T_3 , albumin and UltrosorG and in the presence of 1 nM insulin.

Bile acid uptake assay: This was carried out as described previously [24].

Quantitative RT-PCR analysis: RNA was isolated from livers by homogenization in Trizol reagent (Invitrogen) according to the manufacturer's instructions, followed by purification on an RNeasy column (Qiagen). 2 μg RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's instructions and quantitative PCR was done with SensiMix Plus SYBR kit (Quantace) using Opticon DNA Engine. All primers used are shown in table 1. All values are shown relative to the expression of *cyclophilin b*.

Hepatic glucose output measurement: 16 h after isolation hepatocytes were transferred into glucose-free DMEM (Sigma) containing 2 mM sodium pyruvate and 20 mM lactate. Aliquots of media were removed for glucose measurement at the stated times using TRACE-DMA glucose oxidase kit according to the manufacturers instructions.

Statistical analysis: All data are presented as means \pm SEM. In order to determine statistical significance unpaired, two tailed student t-test was performed for analysis of two groups, whereas data involving more than two groups were assessed by analysis of variance (ANOVA). Statistically significant differences from wild-type was considered for p value < 0.05 .

Results**Generation of liver-specific LKB1 knockout mice**

We crossed mice expressing Cre-recombinase under the control of the albumin promoter [25] with mice harbouring floxed alleles of *Lkb1* [14] to generate *Cre^{+/-}Lkb1^{fl/fl}* animals lacking LKB1 expression in the liver (hereafter referred to as LLKB1KO mice). PCR for the recombination event in a range of tissues demonstrated deletion of *Lkb1* specifically in the liver (Fig. 1A). LKB1 protein expression was not detectable in liver extracts from 15 day old LLKB1KO mice (Fig. 1B). Hepatic LKB1 activity was barely detectable following birth and reached minimal levels by postnatal day 15 (Fig. 1C), confirming functional deletion of LKB1 by 15 days of age. LKB1 protein expression and activity were also reduced in livers from *Lkb1^{fl/fl}* mice (Fig. 1B, C). These findings are consistent with a previous study [14] and arise due to the nature of the targeting event leading to a hypomorphic effect in several tissues. AMPK activity assayed in immune-complexes isolated from LLKB1KO liver extracts was drastically reduced relative to wild-type activity (Fig. 1D). At 15 days the activity of AMPK α 1 containing complexes was reduced by approximately 90% and α 2 activity by >95% in LLKB1KO liver compared to the activity in liver extracts from wild-type mice (Fig. 1D). There was also a slight reduction in AMPK activity in liver extracts isolated from *LKB1^{fl/fl}* mice, although this did not reach statistical significance for α 2 complexes (Fig. 1D). In previous studies, it was reported that deletion of LKB1 in skeletal muscle [26] or heart [27] resulted in loss of AMPK α 2 activity, but α 1 activity was only partially reduced, or was upregulated. Given the results obtained here in liver, the contribution of LKB1 to activation of AMPK α 1 appears to vary depending on the tissue type. LLKB1KO mice were noticeably smaller than either *Lkb1^{fl/fl}* or wild-type mice and had significantly lower body weight from 4 days of age and rapidly began to lose weight from around 15 days of age (Fig. 1E). In contrast, the weight of livers isolated from LLKB1KO mice aged 15 days was significantly increased compared to wild-type mice (0.39 g \pm 0.045 g (LLKB1KO) vs 0.28 g \pm 0.008 g (wild-type), n = 9) This hepatomegaly resulted in a significant increase in the ratio of liver:body weight (Fig. 1F). By 12 days of age LLKB1KO mice started to die and no LLKB1KO mice survived beyond 30 days of age (data not shown). We have been unable to find any obvious metabolic or growth phenotype for *Lkb1^{fl/fl}*, even though there is a significant decrease in LKB1 activity in a number of tissues in these mice compared to wild-type animals. One notable exception is that the male *Lkb1^{fl/fl}* mice are infertile due to a defect in spermatogenesis, which has been described in a previous study [28].

LKB1 deletion leads to disrupted canalicular membranes and defective bile duct formation.

In order to explore the increased mortality of the LLKB1KO mice we undertook histological examination of the liver. No gross morphological changes were apparent and the LKB1KO mouse had a patent common bile duct. In livers from LLKB1KO mice as young as 4 days old, there were increased bile ductular profiles but no well formed ducts around the portal tracts. This observation becomes more apparent with increasing age, as shown in Fig. 2A for liver isolated from a 15 day old animal. Identification of normal bile ducts was difficult in LLKB1KO tissue from mice 15 days or older, whereas bile ducts were clearly visible in livers from wild-type animals at an equivalent age (Fig. 2A-C). Immuno-staining with either osteopontin, a protein excreted by biliary epithelial cells, (Fig. 2B) or CK19, a biliary cytokeratin, (Fig. 2C) showed staining in morphologically normal bile ducts in wild-type liver (marked * in Fig. 2). Although there was staining in liver from LLKB1KO mice, this was associated with the increased bile ductular profiles (marked with an arrow in Fig. 2) and confirmed the lack of

morphologically normal bile ducts. The expression of radixin, a protein usually located at hepatic canalicular membranes, was clearly reduced in LLKB1KO livers (Fig. 2D). In contrast, vascular staining in LLKB1KO mice appeared normal (marked 'v' in Fig. 2 and data not shown). Transmission electron microscopy (TEM) revealed ordered open canalicular channels (c) with well-defined microvilli (mv) in sections of wild-type livers (Fig. 3A). In LLKB1KO livers, abnormal canaliculi were apparent with microvilli often appearing "glued" together from as young as 6 days old. An example of a 15 day old mouse liver is shown in Fig. 3B. In order to determine liver function, serum levels of several liver markers were measured. By 15 days of age serum levels of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) were all significantly raised in LLKB1KO mice (Fig. 3C) whereas albumin levels were lower compared to wild-type (Fig. 3D). By 15-20 days there were patchy areas of necrosis within LLKB1KO livers (Fig. 3E). No significant differences were detected in the LKB1^{fl/fl} mice compared to wild-type mice (Fig. 3 and data not shown).

LLKB1KO mice display marked changes in cholesterol and bile acid metabolism.

Given the abnormal liver architecture and in particular the lack of normal bile ductule formation in the LLKB1KO mice we investigated cholesterol and bile acid metabolism in these animals. Serum cholesterol levels were significantly elevated in LLKB1KO by 8 days of age and continued to rise with increasing age (Fig. 4A). In wild-type mice only 15% of circulating cholesterol was unesterified whereas in LLKB1KO mice more than 50% was unesterified (Fig. 4B). Measured at 15 days of age, total cholesterol was significantly higher and LDL-cholesterol was 10-fold higher in LLKB1KO mice compared to wild-type or Lkb1^{fl/fl} mice, whereas high-density lipoprotein (HDL)-cholesterol and triglyceride levels were not significantly changed (Fig. 4C). There was a significant increase in the concentration of serum bile acids by 4 days of age in LLKB1KO mice relative to wild-type mice (Fig. 4D). The increase in serum bile acids preceded elevation of cholesterol levels (compare Figs. 4A and D). Levels of bile acids in the livers of LLKB1KO mice were very high compared to wild-type, albeit with widely varying levels detected in individual mice (Fig. 4E). The rate of [³H]-taurocholate uptake in isolated hepatocytes from LLKB1KO mice was five fold lower than wild-type cells (Fig. 4F) which could contribute to the elevated levels of serum bile acids. In order to confirm that the hepatocytes isolated from the LLKB1KO animals are viable we measured glucose output in these cells. Hepatic glucose output was increased in the LLKB1KO cells compared to wild-type cells under basal conditions (Fig. 4G). This result is consistent with a previous study in which LKB1 was deleted in adult hepatocytes [29] and demonstrates that the hepatocytes isolated from LLKB1KO mice are metabolically competent to undergo gluconeogenesis, confirming their viability.

We went on to measure the expression of a number of genes known to play a role in bile acid transport and homeostasis (Fig. 5A). There was a significant change in some of the genes normally regulated by elevated levels of bile acids. The genes encoding the basolateral transporters OATP1 and NTCP were down-regulated, whereas expression of the gene encoding the basolateral efflux transporter, MRP4 was increased. However some of the genes expected to be altered by FXR activation due to high levels of bile acids such as genes encoding SHP, CYP7A1, ABCG5/8, MRP3 and BSEP were not significantly altered. In addition, we found a decrease in the expression of MRP2, which encodes a canalicular anion transporter, whereas other studies have found an increase in expression in response to elevated bile acids. Despite the increase in cholesterol levels in the LLKB1KO mice there were no significant changes in

expression of a number of genes involved in cholesterol biosynthesis (Fig. 5B) suggesting that there is no overall upregulation in cholesterol synthesis. Taken together these findings led us to investigate whether the underlying cause of hepatic bile acid accumulation is a defect in bile acid clearance. This would be consistent with the lack of open bile ductules revealed by our histological analyses (Fig. 2).

Defective targeting of BSEP in LLKB1KO mice.

Since our data indicate that increased bile acids in the LLKB1KO mice result from defective clearance we examined the expression of BSEP, one of the key components of bile acid efflux in the liver. BSEP protein expression was unchanged in LLKB1KO livers, as measured by Western blotting (data not shown). We therefore extended our analysis to determine localization of BSEP protein. In wild-type mice, as expected, BSEP was localized predominantly at the canalicular membrane (Fig. 6A). In marked contrast, in liver from LLKB1KO mice, BSEP was found mainly within the cytoplasm, appearing to be associated with cytoplasmic vesicles (Fig. 6A and B). There was no obvious difference in localization of NTCP, a basolateral membrane protein (Fig. 6C). However we observed lower NTCP staining in the LLKB1KO livers compared to wild-type sections.

Loss of hepatic LKB1 leads to abnormal red blood cells and hyperbilirubinemia.

Examination of red blood cells in LLKB1KO mice at 18 days of age showed an abnormal morphology, exhibiting a “spiky” appearance (Fig. 7A). This altered morphology is similar to that described for spur cells of patients with severe liver disease [30]. The red blood cells from LLKB1KO mice were also shown to be more resistant to changes in osmolarity (Fig. 7B). The packed red blood cell volume of LLKB1KO mice was significantly lower than that in wild-type mice (Fig. 7C) implying that the LLKB1KO mice could be anemic. Circulating levels of bilirubin, a product of haem breakdown, were dramatically raised in LLKB1KO mice leading to bright yellow colouration of the serum (Fig. 7D). Bilirubin in either wild-type or LKB1^{fl/fl} serum was virtually undetectable whereas in LLKB1KO mice the levels were hugely elevated, the majority of which was conjugated which shows some functionality of the liver in the older mice.

Discussion

We show here that mice lacking expression of LKB1 in the liver have profound abnormalities in liver architecture and cell morphology that result in severe defects in bile and cholesterol metabolism. Although LKB1 activity was reduced in the liver of LKB1^{fl/fl} mice there was no hepatic phenotype, suggesting that there is significant “spare capacity” within the signaling pathway, and that LKB1 is not rate-limiting. This may be a more general feature of protein kinase signaling pathways, for example mice expressing 10% of the normal levels of PDK1 appear normal, whereas complete deletion results in embryonic lethality [31]. Our data show that LKB1 is required for the normal postnatal development of the biliary system including correct localization of canalicular membrane proteins and development of bile ducts. The molecular mechanisms underlying development of the biliary tree are poorly understood. A number of other studies have demonstrated that LKB1 plays a key role in determining polarity in mammalian cells as well as in other model organisms [32]. Activation of LKB1 was shown to cause complete polarization of single mammalian epithelial cells [33]. An attractive hypothesis is that LKB1 is required for polarization of hepatocytes and that disruption of the normal development of the bile canaliculi in the LLKB1KO mice is a consequence of a defect in polarity

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determination. In support of this, we found that BSEP and radixin was no longer targeted to the canalicular membrane in the absence of LKB1. The failure to thrive and loss of weight of LLKB1KO mice may be caused by poor absorption of nutrients from the intestine due to the inability to transport bile from the liver via the biliary tree, which is defective in these animals. Associated with the marked anatomical abnormalities seen in LLKB1KO mice we found a number of metabolic defects. There was a dramatic increase in serum and liver bile acids which stems from defective clearance of bile acids into the canaliculus as a direct consequence of mislocalisation of BSEP. Previous studies have shown that bile acids regulate the transcription of genes encoding proteins involved in bile acid metabolism and transport. However, in spite of elevated circulating bile acids some of the genes known to be transcriptionally controlled by FXR [34] were not altered in LLKB1KO liver. We observed a decrease in the expression of the basolateral transporters *Oatp* and *Ntcp*, as well as decreased staining of NTCP in the livers from LLKB1KO mice, which would explain a decrease in the taurocholate uptake in the isolated hepatocytes from LLKB1KO animals (Fig. 4F). However, the expected transcriptional up regulation of those transporters in the canalicular membrane e.g. *Bsep*, *ABCB5/8*, *Mrp2* is not apparent in the LLKB1KO livers. There appears to be a lack of control of those genes involved in expression of proteins located to the canalicular membrane. Expression of the gene encoding CYP7A1, the rate-limiting enzyme in bile acid synthesis, is not decreased in response to the elevated bile acids in the LLKB1KO livers. The intestine as well as the liver plays a critical role in bile acid homeostasis. It has been shown that in response to bile acids in the intestine FXR stimulates fibroblast growth factor 15 (FGF15) expression in the intestine which signals via FGF receptor 4 (FGFR4) to bring about down regulation of CYP7A1 in the liver. [35]. However, this route of control is likely to be defective in the LLKB1KO mice because bile acids are not flowing into the bile for release into the intestine, and so cannot activate intestinal FXR. In this case, transcriptional suppression of CYP7A1 by FGF15 signalling would not be expected to occur, which may explain the modest decrease in expression that we observe in LLKB1KO liver. Dramatically elevated levels of circulating, unesterified cholesterol were observed in the LLKB1KO mice which, is most likely a secondary effect resulting from the reduced clearance of bile acids. It has been reported that cholestasis results in the appearance of a specific form of lipoprotein, lipoprotein-X (LP-X), which has a high content of unesterified cholesterol and unusual protein content [36, 37]. It is likely that LP-X makes up a proportion of the elevated LDL observed in the LLKB1KO. An attractive hypothesis is that the increased circulating free-cholesterol leads to the defect in red blood cells that we observed in the LLKB1KO mice. Supporting this is a previous study that has shown that high circulating free cholesterol can alter the composition of the erythrocyte membrane [38]. Excess membrane cholesterol leads to characteristic morphological abnormalities and formation of spur cells. The altered lipid composition of the red blood cell membrane results in spur cells with decreased deformability, which is essential for passage through capillary beds [39]. Destruction of the spur cells by the spleen ultimately leads to anaemia as the bone marrow cannot produce sufficient new cells to compensate for the loss. The increased destruction of erythrocytes leads to a further increase in circulating bilirubin, which cannot be appropriately disposed of in the bile due to defective canaliculi and bile ducts thereby leading to excessive accumulation in the serum. It is apparent that the liver is able to conjugate the majority of the excess bilirubin, which shows the liver, even in late stages, maintains some functionality and the hyperbilirubinemia is likely due to impaired transport and export from the liver. In a previous study it has been shown that radixin deficiency causes conjugated hyperbilirubinemia with loss of *Mrp2* (the major transporter of bilirubin) from

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the canalicular membranes [40]. As radixin is depleted in LLKB1KO this is a possible explanation for elevated levels of conjugated bilirubin.

In a previous study in which LKB1 expression was deleted in adult liver [15] there was no report of altered bile acid or cholesterol metabolism. One possible reason for the absence of this phenotype could be that LKB1 activity was not completely abolished in that model, and that this might reduce the severity of the subsequent phenotype. However, an alternative explanation is that in adult livers, biliary tree formation would have progressed normally through development, precluding the onset of the phenotype we describe in our model.

The lack of open tubular bile ducts and areas of necrosis in the postnatal livers seen in LLKB1KO livers are akin to observations seen in murine models of Alagille syndrome which are due to alterations in Notch signalling [41, 42]. As in Notch 2 inactivation, loss of LKB1 activity results in defective intrahepatic bile duct development. However, to date there has been no direct connection between Notch and LKB1 signaling pathways made, though further investigation maybe warranted.

In summary, we have shown here a key role for LKB1 in coordinating the localization of canalicular membrane proteins and canalicular formation. The lack of LKB1 leads to the inability of the liver to transport and dispose of constituents of bile, resulting in a toxic build up of circulating bile acids, bilirubin and unesterified cholesterol. It is noteworthy that deletion of both AMPK α 1 and α 2 in the liver does not result in a similar phenotype to the current model [43] but a very recent study in isolated hepatocytes reported a role for LKB1 and AMPK in the formation and maintenance of the canalicular network [44]. This finding supports a role for LKB1, AMPK and AMPK-related kinases in bile acid metabolism and canalicular formation. Further studies aimed at investigating the role of AMPK and the AMPK-related kinases downstream of LKB1 in hepatic development and liver disease is warranted. In summary, our study uncovers a new role for LKB1 in the liver adding to the list of diverse functions of this master regulatory kinase.

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Table 1

Sequences of the oligonucleotide primers used for qRT-PCR.

mRNA	Forward 5'-3'	Reverse 5'-3'
<i>ABCG5</i>	TGGGTCCCAAGGAGTATGC	GCTCCAAGACTTCACACAGTG
<i>ABCG8</i>	AGTGGTCAGTCCAACACTCTG	GAGACCTCCAGGCTATCTTGAA
<i>BSEP</i>	GGGAGCAGTGGGTGTGGTAAAG	TCCTGGGAGACAATCCCAATGTT
<i>cyclophilin b</i>	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT
<i>CYP7A1</i>	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
<i>FXR</i>	GGCAGAATCTGGATTGGAATCG	GCTGAACCTGAGGAAACGGG
<i>HMGCoAR</i>	GATTCTGGCAGTCAGTGGGAA	GTTGTAGCCGCCTATGCTCC
<i>MRP2</i>	ATGAAGTGACAGAGGGCGGT	TGCAGCCTGTGTGCGATAG
<i>MRP3</i>	GCAGCAGAACCAAGCATCAAG	GACCGCATCCTCACCTGG
<i>MRP4</i>	GGTTGGAATTGTGGGCAGAA	TGGTCCGTGTGGTCATTGAA
<i>NTCP</i>	CTGCCGCCTGGCTTTGGCCA	CTGGAGCAGGTGGTCATCAC
<i>OATP1</i>	TGATACACGCTGGGTCGGTG	GCTGCTCCAGGTATTTGGGC
<i>shp</i>	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
<i>SREBP1a</i>	GTTGATGAGCTGGAGCATGT	CTCCCTCCTTACCCTTGGAG
<i>SREBP1c</i>	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
<i>SREBP2</i>	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA

Figure Legends

Figure 1

Liver-specific deletion of *Lkb1*. (A) DNA from tissues of 15 day old mice and recombination of the floxed *Lkb1* allele detected by PCR. The recombination product is indicated by an arrow. Interleukin-2 (IL-2) was used as an internal control. (B) LKB1 immune-complexes from liver homogenates blotted for LKB1. (C) LKB1 activity measured in duplicate immune-complexes from liver homogenates (n = 3). (D) AMPK activity was measured in AMPK α 1 or α 2 specific immune-complexes isolated from liver homogenates of 15 day old mice (n = 5). Results are expressed as units/mg of total protein where 1U=1 nmole of $^{32}\text{PO}_4$ incorporated into SAMS peptide per min. (E) Body weights of wild-type or LLKB1KO mice are shown at the indicated days after birth (n = 9-11). (F). Ratio of liver weight:body weight. Results are shown as the mean \pm SEM. Statistically significant differences from wild-type are shown by * (p < 0.05), ** (p < 0.01) and *** (p < 0.001).

Figure 2

Altered liver architecture in LLKB1KO livers. Livers of 15 day old wild-type (Wt) and LLKB1KO mice were stained with Hematoxylin-eosin (A), immunostained with osteopontin (B), CK19 (C) or radixin (D). Arrows indicate increased bile ductular profiles and no morphologically normal open bile ducts in LLKB1KO mice. Normal bile ducts are marked * and blood vessels labeled 'v' in each case. A representative example of at least 3 livers is shown.

Figure 3

Altered canalicular morphology and liver function in LLKB1KO mice. Transmission electron micrographs of wild-type (A) and LLKB1KO (B) livers showing canalicular membranes of 15 day old mice. Canalicular channel is labelled (c), microvilli (mv) and tight junctions (tj). A representative example of at least 3 livers is shown. (C) Serum levels of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) and (D) albumin from 15 day old wild-type and LLKB1KO mice. Results are the mean values \pm SEM from $n = 5$ mice per genotype. Statistically significant differences from wild-type values are shown by * ($p < 0.05$), ** ($p < 0.01$). (E) Toluene blue stained liver section from an 18 day old LLKB1KO mouse showing patchy necrosis. This is a representative image of 3 livers examined.

Figure 4

Lack of hepatic LKB1 disrupts cholesterol and bile acid metabolism. (A) Total serum cholesterol over time (age shown in days) ($n = 4-6$). (B) Unesterified cholesterol as a percentage of total serum cholesterol in 15 day old mice ($n = 4-6$). (C) Total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride concentrations from serum of 15 day old mice are shown ($n=7-10$). (D) Serum bile acids concentrations over time (age in days) ($n = 4-6$). (E) Bile acids measured in liver extracts from wild-type (Wt) and LLKB1KO mice with the mean value shown by a bar ($n = 5-6$). (F) [3 H]Taurocholate (TC) uptake was measured in hepatocytes isolated from 15 day old wild-type or LLKB1KO mice. Uptake is shown relative to wild-type cells. Triplicate measurements of 3 independent experiments. In each case, results are shown as the mean \pm SEM and statistically significant differences from wild-type are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Figure 5

Expression of genes involved in bile acid and cholesterol homeostasis.

Expression levels of genes in livers of 15 day old wild-type (Wt) or LLKB1KO mice measured by qRT-PCR for genes involved in bile acid homeostasis (A) or cholesterol synthesis (B). The results are shown as relative expression normalized to *cyclophilin b* and the ratio of expression in LLKB1KO livers is shown compared to wild-type values set as 1 ($n = 6$). Results are shown as the mean \pm SEM and statistically significant differences from wild-type are indicated by * ($p < 0.05$), ** ($p < 0.01$).

Figure 6

Cytoplasmic localization of BSEP in LLKB1KO livers. (A) Immunofluorescence staining for BSEP in 15 day old mice with (B) magnification of the indicated areas shown in A. (C) Immunostaining of NTCP in livers from 15 day old mice. A representative example of at least 3 livers is shown.

Figure 7

Development of hyperbilirubinemia in LLKB1KO mice. (A) Semi-thin sections from wild-type or LLKB1KO livers showing erythrocytes inside blood vessels of 18 day old mice. A representative example of 3 livers of mice aged 18-21 days is shown. (B) Osmotic fragility of wild-type and LLKB1KO erythrocytes isolated from 15 day old mice. Relative haemolysis compared to 100% in pure water ($O_s = 0$) ($n = 6$). (C) Haemocrit values of 15 day old mice ($n = 6$). (D) Serum levels of conjugated and unconjugated bilirubin of LLKB1KO mice of different ages (shown in days) ($n = 5$). Insert shows marked yellow appearance of LLKB1KO serum compared to wild-type. Results are the mean \pm SEM statistically significant differences from the wild-type values are shown by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Figure 1

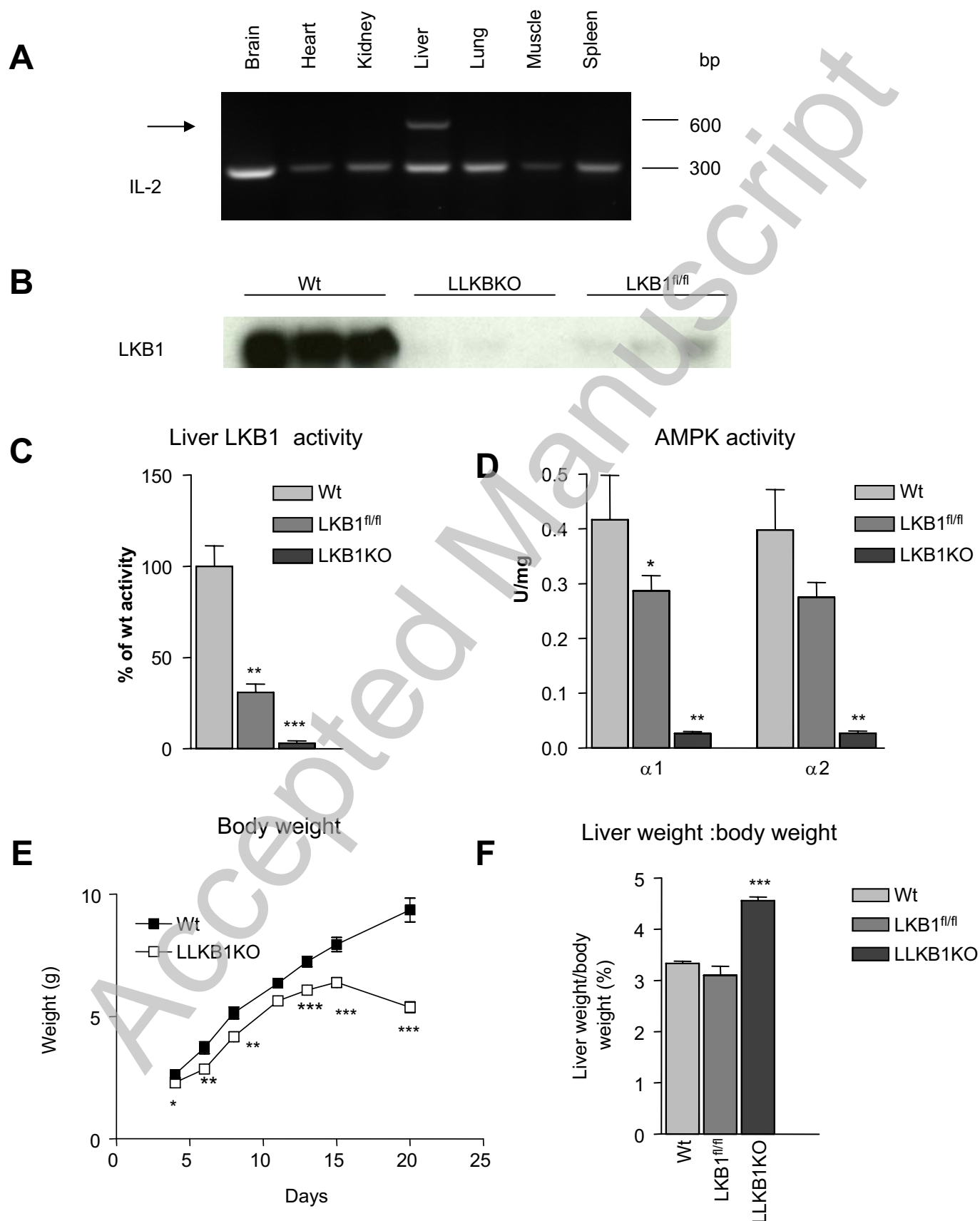


Figure 2

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20101721

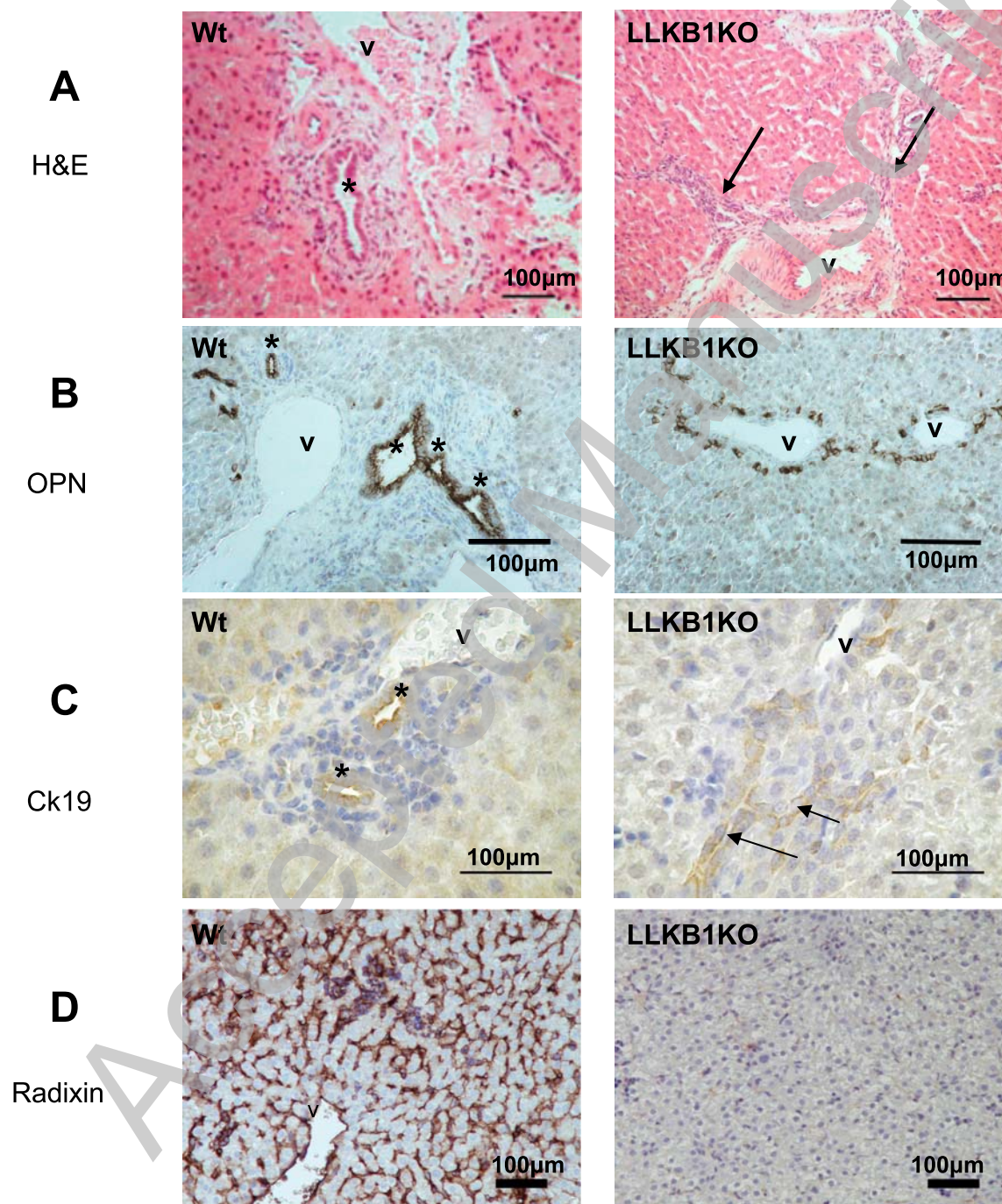


Figure 3

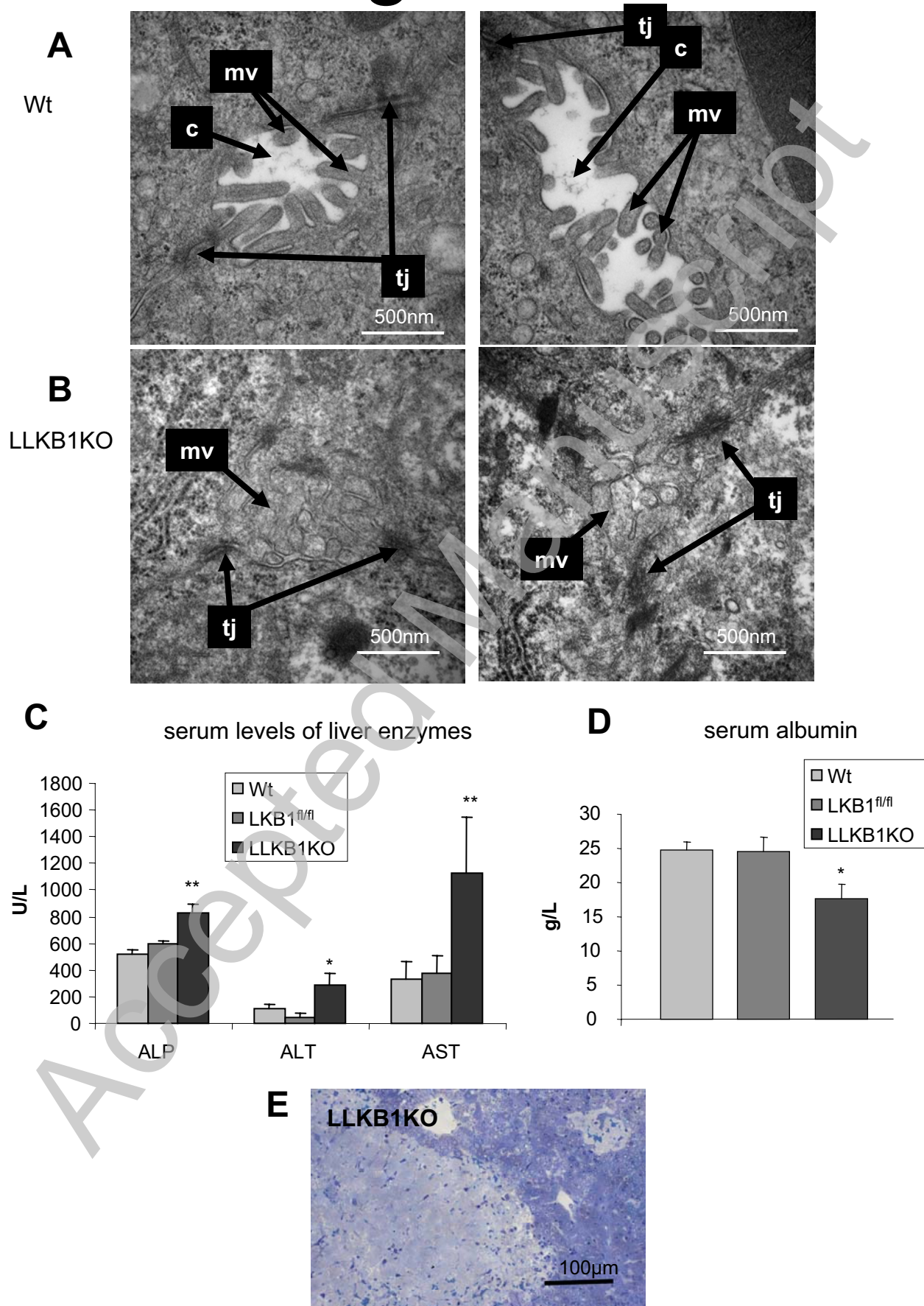


Figure 4

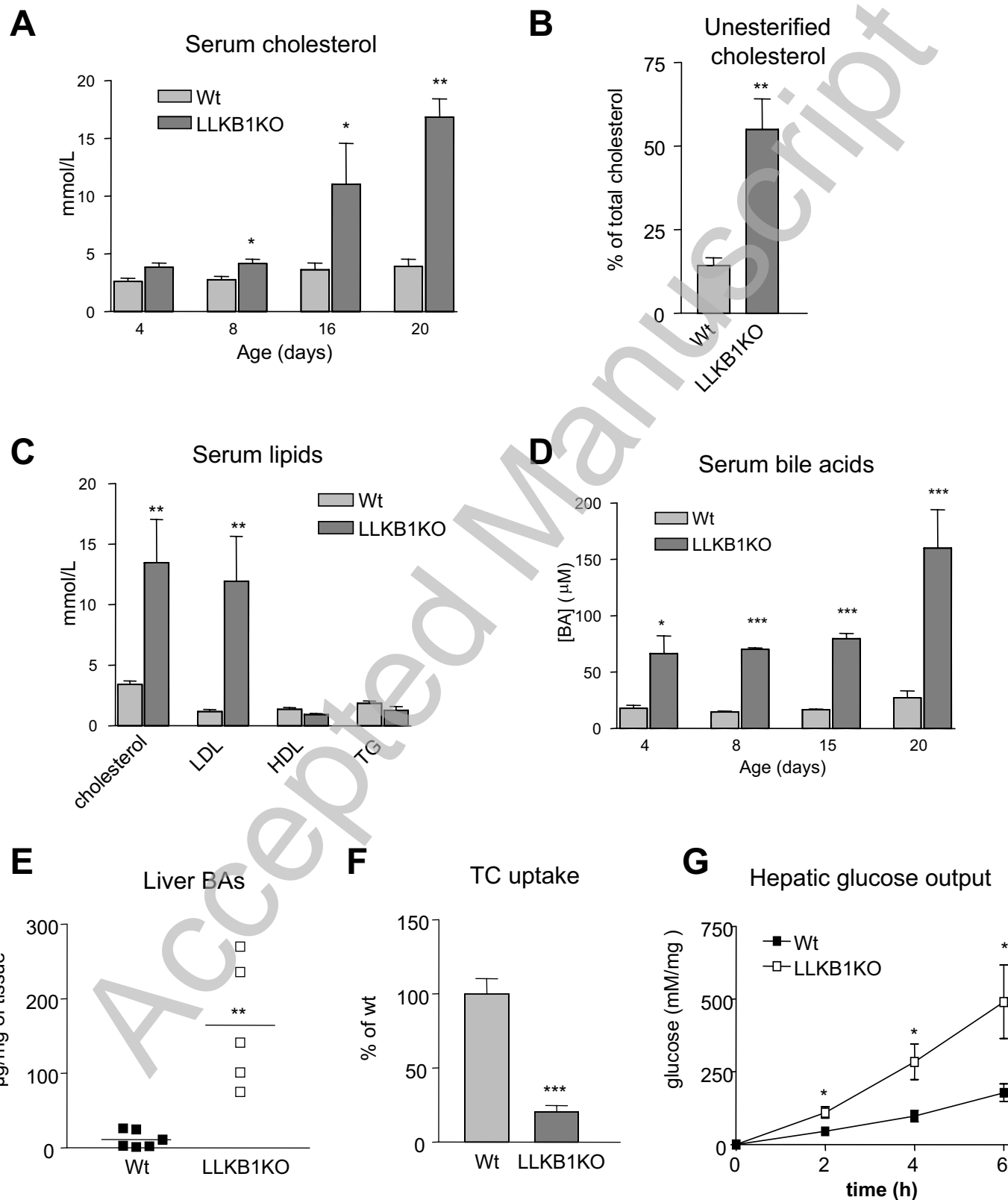
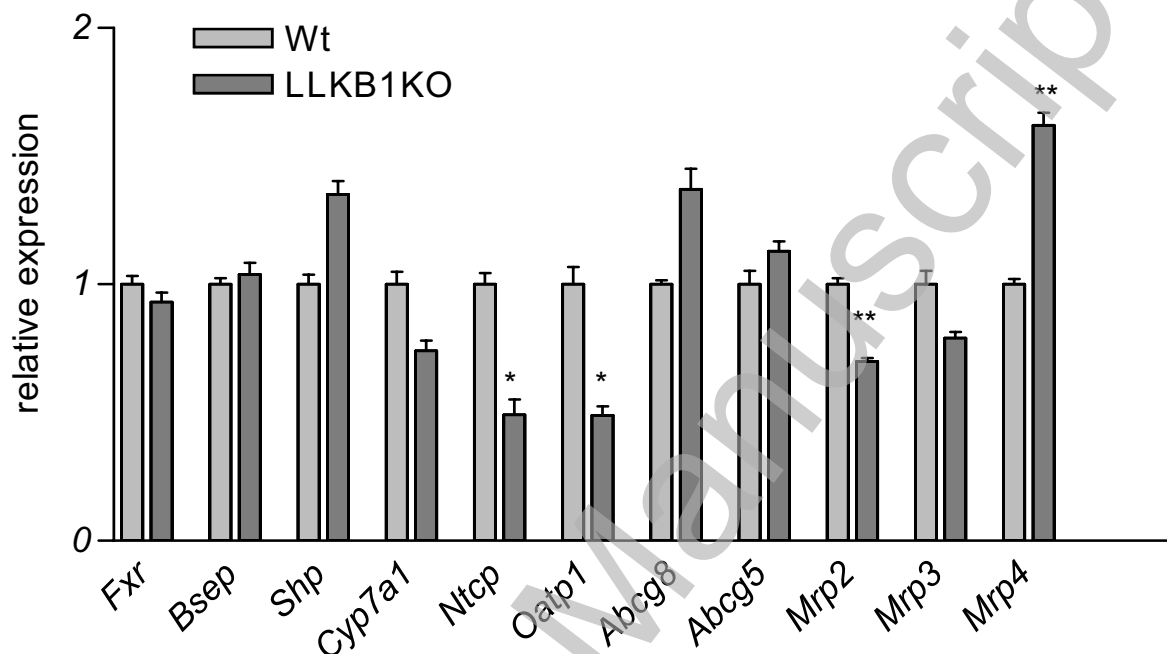


Figure 5

A

Bile acid homeostasis gene expression



B

Cholesterogenic gene expression

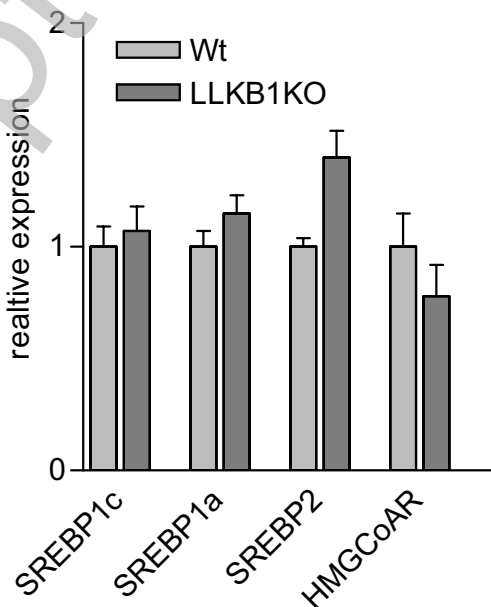
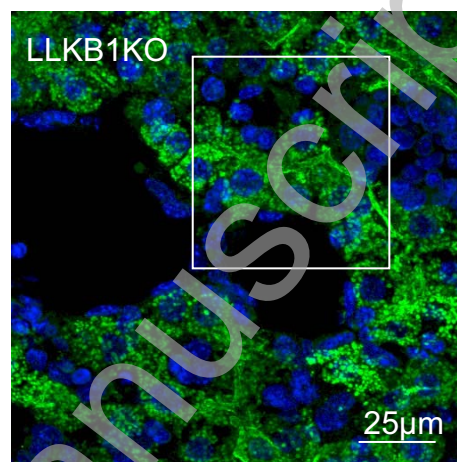
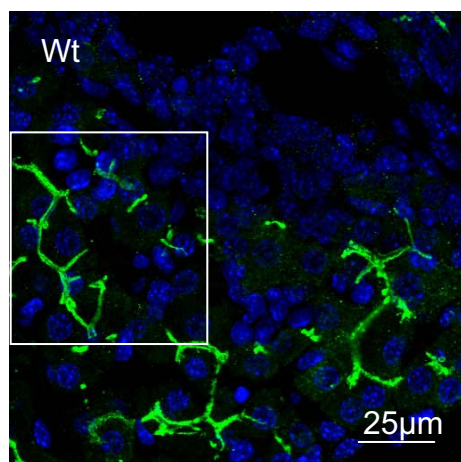
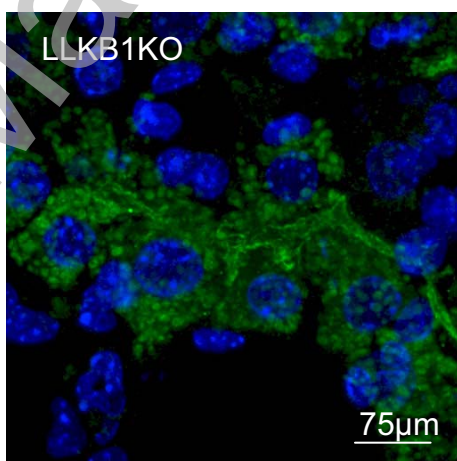
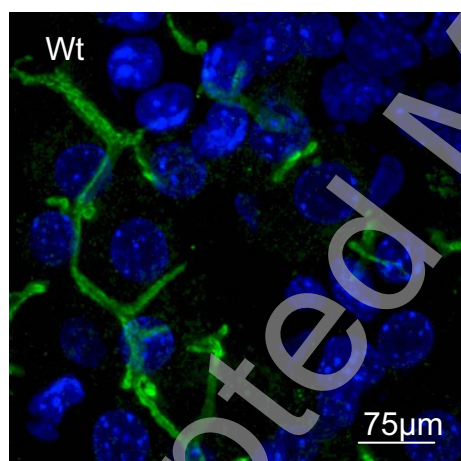


Figure 6

A
BSEP



B
BSEP zoom



C
NTCP

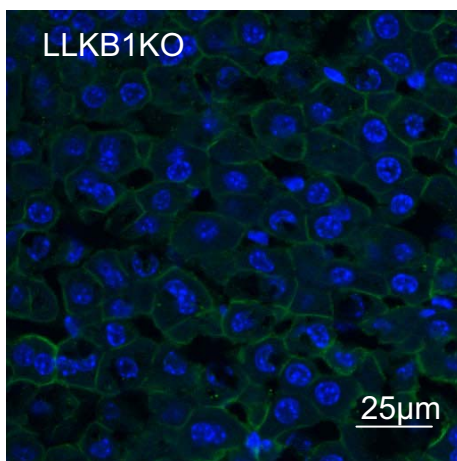
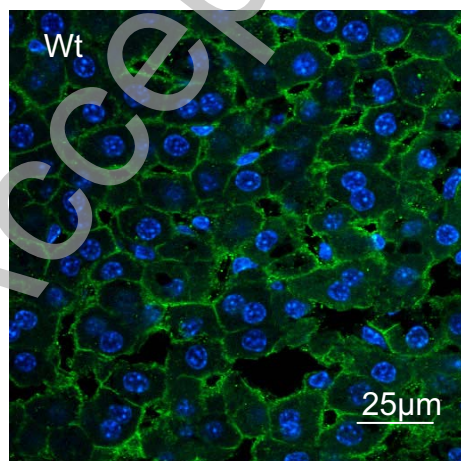
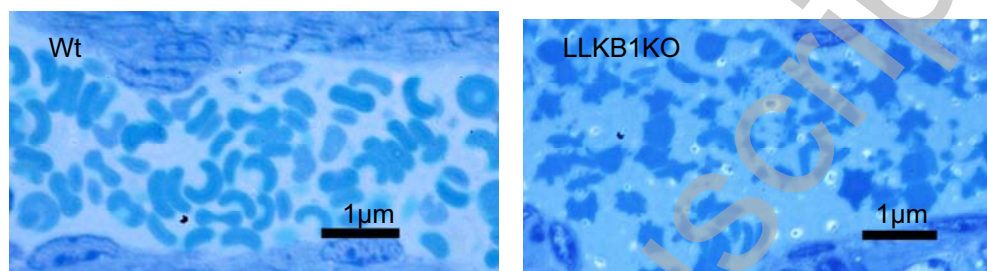


Figure 7

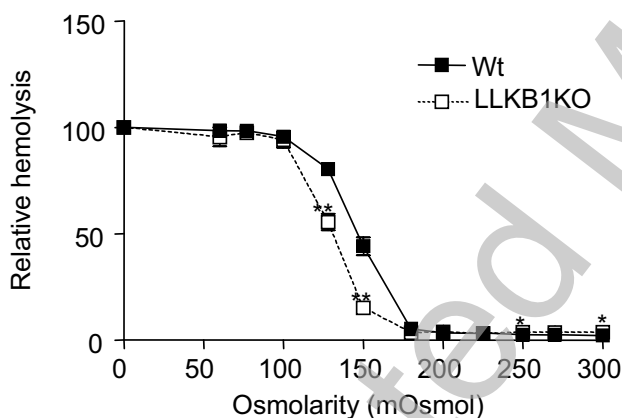
A

Red blood cells



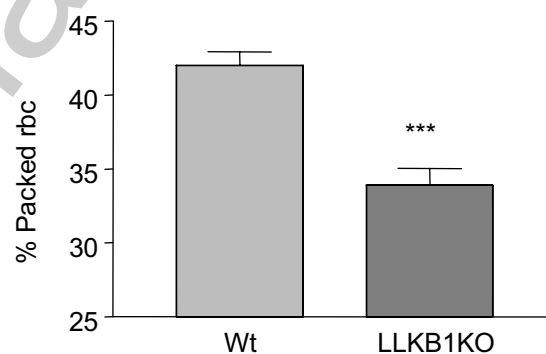
B

Osmotic fragility



C

Haemocrit



D

Serum bilirubin measurements
in LLKB1KO mice

